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=> s nerve growth factor
L1        36485 NERVE GROWTH FACTOR

=> s proNGF or preproNGF
L2           82 PRONGF OR PREPRONGF

=> s l2 and (natur##### or denatur##### or renatur#####)
L3           36 L2 AND (NATUR##### OR DENATUR##### OR RENATUR#####)

=> duplicate remove
ENTER L# LIST OR (END):l3
DUPLICATE PREFERENCE IS 'MEDLINE, BIOSIS, USPATFULL, PCTFULL'
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PROCESSING COMPLETED FOR L3
L4            34 DUPLICATE REMOVE L3    (2 DUPLICATES REMOVED)

=>

=> d 1-34

L4   ANSWER 1 OF 34  USPATFULL
AN     2003:57518  USPATFULL
TI     Mutant pro-neurotrophin with improved activity
IN     Tuszynski, Mark, La Jolla, CA, UNITED STATES
       Blesch, Armin, San Diego, CA, UNITED STATES
PI     US 2003040082      A1   20030227
AI     US 2001-788188     A1   20010216 (9)
DT     Utility
FS     APPLICATION
LN.CNT 628
INCL   INCLM: 435/069.400
       INCLS: 424/093.210; 435/325.000; 536/023.500; 530/399.000; 435/320.100
NCL    NCLM:  435/069.400
       NCLS:  424/093.210; 435/325.000; 536/023.500; 530/399.000; 435/320.100
IC     [7]
       ICM: A61K048-00
       ICS: C07H021-04; C12N005-06; A61K038-18; C07K014-48

L4   ANSWER 2 OF 34  USPATFULL
AN     2002:251935  USPATFULL
TI     Purification of NGF
IN     Burton, Louis E., San Mateo, CA, UNITED STATES
       Schmelzer, Charles H., Burlingame, CA, UNITED STATES
       [illegible]
```

FS APPLICATION
LN.CNT 2052
INCL INCLM: 530/350.000
INCLS: 530/417.000
NCL NCLM: 530/350.000
NCLS: 530/417.000
IC [7]
ICM: C07K014-435
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L4 ANSWER 3 OF 34 USPATFULL
AN 2002:85534 USPATFULL
TI NOVEL NEUROTROPHIC FACTOR
IN ROSENTHAL, ARNON, PACIFICA, CA, UNITED STATES
PI US 2002045576 A1 20020418
US 6506728 B2 20030114
AI US 1995-450842 A1 19950526 (8)
RLI Division of Ser. No. US 1995-426419, filed on 19 Apr 1995, ABANDONED
Continuation of Ser. No. US 1993-30013, filed on 22 Mar 1993, ABANDONED
A 371 of International Ser. No. WO 1991-US6950, filed on 24 Sep 1991,
UNKNOWN Continuation-in-part of Ser. No. US 1991-648482, filed on 31 Jan
1991, ABANDONED Continuation-in-part of Ser. No. US 1990-587707, filed
on 25 Sep 1990, GRANTED, Pat. No. US 5364769
DT Utility
FS APPLICATION
LN.CNT 2815
INCL INCLM: 514/012.000
INCLS: 514/002.000
NCL NCLM: 514/012.000
NCLS: 514/002.000
IC [7]
ICM: A01N037-16
ICS: A61K038-17
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L4 ANSWER 4 OF 34 USPATFULL
AN 2002:254170 USPATFULL
TI Assays for promoter operability in central nervous system cells
IN Kahn, Axel, Paris, FRANCE
Le Gal la Salle, Gildas, Saint Cloud, FRANCE
Mallet, Jacques, Paris, FRANCE
Perricaudet, Michel, Ecrosnes, FRANCE
Peschanski, Marc, Creteil, FRANCE
Robert, Jean-Jacques, Sceaux, FRANCE
PA Aventis Pharma S.A., Antony, FRANCE (non-U.S. corporation)
PI US 6458529 B1 20021001
AI US 1995-459994 19950602 (8)
RLI Continuation of Ser. No. US 1993-403868, filed on 17 Sep 1993
PRAI EP 1992-402644 19920925
DT Utility
FS GRANTED
LN.CNT 1947
INCL INCLM: 435/006.000
INCLS: 435/007.910; 435/455.000; 435/320.100; 424/093.100; 424/093.200
NCL NCLM: 435/006.000
NCLS: 424/093.100; 424/093.200; 435/007.910; 435/320.100; 435/455.000
IC [7]
ICM: C12Q001-68
ICS: C12N015-63; C12N015-00; A01N063-00
[illegible]

L4 ANSWER 5 OF 34 USPATFULL
TI Isolation of neurotrophins from a mixture containing them by [illegible]
neurotrophin variants using hydrophobic interaction chromatography
[illegible]

Beck, Joanne T., Westlak[redacted]illage, CA, United States
PA Genentech, Inc., So. San [redacted]ancisco, CA, United States (U.S[redacted] corporation)
PI US 6423831 B1 20020723
AI US 2000-675503 20000929 (9)
RLI Continuation of Ser. No. US 1999-363573, filed on 29 Jul 1999, now patented, Pat. No. US 6184360 Continuation of Ser. No. US 1997-970865, filed on 14 Nov 1997, now patented, Pat. No. US 6005081
PRAI US 1997-47855P 19970529 (60)
US 1996-30838P 19961115 (60)
DT Utility
FS GRANTED
LN.CNT 2348
INCL INCLM: 530/399.000
INCLS: 530/324.000; 530/350.000; 530/412.000; 530/416.000; 530/417.000; 435/069.100; 435/069.400; 435/070.100; 435/071.100
NCL NCLM: 530/399.000
NCLS: 435/069.100; 435/069.400; 435/070.100; 435/071.100; 530/324.000; 530/350.000; 530/412.000; 530/416.000; 530/417.000
IC [7]
ICM: C07K003-14
ICS: C12P021-06
EXF 530/399; 530/324; 530/350; 530/412; 530/416; 530/417; 435/69.1; 435/69.4; 435/70.1; 435/71.1
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L4 ANSWER 6 OF 34 PCTFULL COPYRIGHT 2003 Univentio
AN 2002096356 PCTFULL ED 20021217 EW 200249
TIEN HIGH AFFINITY LIGAND FOR p75 NEUROTROPHIN RECEPTOR
TIFR LIGAND A FORTE AFFINITE POUR LE RECEPTEUR DE LA NEUROTROPHINE P75
IN HEMPSTEAD, Barbara, L., 525 East 86th Street, Apartment 9c, New York, NY 10028, US [US, US];
LEE, Ramee, 1233 York Avenue, #16E, New York, NY 10021, US [US, US];
TENG, Kenneth, K., 315 West 86th Street, Apartment 8B, New York, NY 10024, US [—, US];
KERMANI, Pouneh, 320 East Shore Road, Apartment 12A, Greak Neck, NY 11023, US [CA, US]
PA CORNELL RESEARCH FOUNDATION, INC., 20 Thornwood Drive, Ithaca, NY 14850, US [US, US], for all designates States except US;
HEMPSTEAD, Barbara, L., 525 East 86th Street, Apartment 9c, New York, NY 10028, US [US, US], for US only;
LEE, Ramee, 1233 York Avenue, #16E, New York, NY 10021, US [US, US], for US only;
TENG, Kenneth, K., 315 West 86th Street, Apartment 8B, New York, NY 10024, US [—, US], for US only;
KERMANI, Pouneh, 320 East Shore Road, Apartment 12A, Greak Neck, NY 11023, US [CA, US], for US only
AG FEIT, Irving, N., Hoffmann & Baron, LLP, 6900 Jericho Turnpike, Syosset, NY 11791, US
LAF English
LA English
DT Patent
PI WO 2002096356 A2 20021205
DS W: AE AG AL AM AT AU AZ BA BB BG BR BY BZ CA CH CN CO CR CU CZ DE DK DM DZ EC EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX MZ NO NZ OM PH PL PT RO RU SD SE SG SI SK SL TJ TM TN TR TT TZ UA UG US UZ VN YU ZA ZM ZW
RW (ARIPO): GH GM KE LS MW MZ SD SL SZ TZ UG ZM ZW
RW (EAPO): AM AZ BY KG KZ MD RU TJ TM
RW [illegible]

L4 ANSWER [illegible] OF 34 PCTFULL COPYRIGHT [illegible]
AN 2002082076 PCTFULL ED 20021028 EW 200242

TIFR PROCEDE DE DEPISTAGE DE [redacted]ADIES DEMENTIELLES CHRONIQUES, [redacted] PEPTIDES ET REACTIFS DE DEPISTAGE CORRESPONDANTS
TIDE VERFAHREN ZUM NACHWEIS CHRONISCH-DEMENZIELLER ERKRANKUNGEN, ZUGEHOeRIGE PEPTIDE UND NACHWEISREAGENZIEN
IN LAMPING, Norbert, Siegesstrasse 8, 30175 Hannover, DE [DE, DE];
ZUCHT, Hans-Dieter, Von-Escherte-Strasse 6, 30539 Hannover, DE [DE, DE];
HEINE, Gabriele, Waldstrasse 22, 30163 Hannover, DE [DE, DE];
JUeRGENS, Michael, Waldstrasse 22, 30163 Hannover, DE [DE, DE];
HESS, Ruediger, Bollnaeser Strasse 2, 30629 Hannover, DE [DE, DE];
SELLE, Hartmut, Eickenriede 15, 30459 Hannover, DE [DE, DE];
KELLMANN, Markus, Heinrich-Stamme-Strasse 3, 30171 Hannover, DE [DE, DE]
PA BIOVISION AG, Feodor-Lynen-Strasse 5, 30625 Hannover, DE [DE, DE], for all designates States except US;
LAMPING, Norbert, Siegesstrasse 8, 30175 Hannover, DE [DE, DE], for US only;
ZUCHT, Hans-Dieter, Von-Escherte-Strasse 6, 30539 Hannover, DE [DE, DE], for US only;
HEINE, Gabriele, Waldstrasse 22, 30163 Hannover, DE [DE, DE], for US only;
JUeRGENS, Michael, Waldstrasse 22, 30163 Hannover, DE [DE, DE], for US only;
HESS, Ruediger, Bollnaeser Strasse 2, 30629 Hannover, DE [DE, DE], for US only;
SELLE, Hartmut, Eickenriede 15, 30459 Hannover, DE [DE, DE], for US only;
KELLMANN, Markus, Heinrich-Stamme-Strasse 3, 30171 Hannover, DE [DE, DE], for US only
AG LAeUFER, Martina, Gramm, Lins & Partner GbR, Freundallee 13, 30173 Hannover, DE
LAF German
LA German
DT Patent
PI WO 2002082075 A2 20021017
DS W: AE AG AL AM AT AU AZ BA BB BG BR BY BZ CA CH CN CO CR CU CZ DE DK DM DZ EC EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX MZ NO NZ OM PH PL PT RO RU SD SE SG SI SK SL TJ TM TN TR TT TZ UA UG US UZ VN YU ZA ZM ZW
RW (ARIPO): GH GM KE LS MW MZ SD SL SZ TZ UG ZM ZW
RW (EAPO): AM AZ BY KG KZ MD RU TJ TM
RW (EPO): AT BE CH CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE TR
RW (OAPI): BF BJ CF CG CI CM GA GN GQ GW ML MR NE SN TD TG
AI WO 2002-DE1376 A 20020408
PRAI DE 2001-101 17 431.4 20010406
ICM G01N033-08

L4 ANSWER 8 OF 34 PCTFULL COPYRIGHT 2003 Univentio
AN 2002066645 PCTFULL ED 20020910 EW 200235
TIEN MUTANT PRO-NEUROTROPHIN WITH IMPROVED ACTIVITY
TIFR PRO-NEUROTROPHINES MUTANTES DOTEES D'UNE ACTIVITE PLUS EFFICACE
IN TUSZYNSKI, Mark, 7508 Mar Avenue, La Jolla, CA 92037, US;
BLESCH, Armin, 4360 Mt. Putman Avenue, San diego, CA 92107, US
PA REGENTS OF THE UNIVERSITY OF CALIFORNIA, Office of Technology Transfer, 5th floor, 1111 Franklin Street, Oakland, CA 94607-5200, US [US, US]
AG TAYLOR, Stacy, L., Foley & Lardner, P.O. Box 80278, San Diego, CA 92138-0278, US
LAF English
LA English
DT Patent
[redacted]

AI WO 2002-US4395 A 20020215
PRAI US 2001-09/788,188 20010216
ICM C12N015-12
ICS C07K014-475; A61K038-17

L4 ANSWER 9 OF 34 PCTFULL COPYRIGHT 2003 Univentio
AN 2002044203 PCTFULL ED 20020624 EW 200223
TIEN PRODUCTION OF RECOMBINANT BMP-2
TIFR PRODUCTION DE BMP-2 RECOMBINEE
TIDE HERSTELLUNG VON REKOMBINANTEM BMP-2
IN RUDOLPH, Rainer, Dr.-Hans-Litten-Str. 28, 06120 Halle, DE [DE, DE];
SCHWARZ, Elisabeth, Dr.-Hans-Litten-Str. 28, 06120 Halle, DE [DE, DE];
HERR, Gerhard, Turmstrasse 16, 35578 Wetzlar, DE [DE, DE];
HILLGER, Frank, Fischereiweg 32, 06846 Dessau, DE [DE, DE]
PA SCIL PROTEINS GMBH, Heinrich-Damerow-Strasse 1, 06120 Halle, DE [DE, DE], for all designates States except US;
RUDOLPH, Rainer, Dr.-Hans-Litten-Str. 28, 06120 Halle, DE [DE, DE], for US only;
SCHWARZ, Elisabeth, Dr.-Hans-Litten-Str. 28, 06120 Halle, DE [DE, DE], for US only;
HERR, Gerhard, Turmstrasse 16, 35578 Wetzlar, DE [DE, DE], for US only;
HILLGER, Frank, Fischereiweg 32, 06846 Dessau, DE [DE, DE], for US only
AG WEICKMANN & WEICKMANN, Postfach 860 820, 81635 Muenchen, DE
LAF German
LA German
DT Patent
PI WO 2002044203 A2 20020606
DS W: AE AG AL AM AT AU AZ BA BB BG BR BY BZ CA CH CN CO CR CU CZ
DE DK DM DZ EC EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP
KE KG KP KR KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX MZ
NO NZ OM PH PL PT RO RU SD SE SG SI SK SL TJ TM TR TT TZ UA
UG US UZ VN YU ZA ZM ZW
RW (ARIPO): GH GM KE LS MW MZ SD SL SZ TZ UG ZM ZW
RW (EAPO): AM AZ BY KG KZ MD RU TJ TM
RW (EPO): AT BE CH CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE TR
RW (OAPI): BF BJ CF CG CI CM GA GN GQ GW ML MR NE SN TD TG
AI WO 2001-EP13840 A 20011127
PRAI DE 2000-100 59 336.4 20001129
ICM C07K014-00

L4 ANSWER 10 OF 34 USPATFULL
AN 2001:18606 USPATFULL
TI Purification of NGF
IN Burton, Louis E., San Mateo, CA, United States
Schmelzer, Charles H., Burlingame, CA, United States
Beck, Joanne T., Westlake Village, CA, United States
PA Genentech, Inc., South San Francisco, CA, United States (U.S. corporation)
PI US 6184360 B1 20010206
AI US 1999-363573 19990729 (9)
RLI Continuation of Ser. No. US 1997-970865, filed on 14 Nov 1997, now patented, Pat. No. US 6005081
PRAI US 1996-30838P 19961115 (60)
US 1997-47855P 19970529 (60)
DT Utility
FS Granted
LN.CNT 2226
INCL INCLM: 530/399.000
INCLS: 530/324.000; 530/350.000; 530/412.000; 530/416.000; 530/417.000;
[illegible]
[illegible]
ICS: [illegible]
EXF [illegible]

L4 ANSWER 11 OF 34 USPATFULL
AN 2001:7868 USPATFULL
TI Neuronal factor
IN Rosenthal, Arnon, Pacifica, CA, United States
Winslow, John W., El Granada, CA, United States
PA Genentech, Inc., So. San Francisco, CA, United States (U.S. corporation)
PI US 6174701 B1 20010116
AI US 1995-455741 19950531 (8)
RLI Continuation of Ser. No. US 1995-381030, filed on 31 Jan 1995
Continuation of Ser. No. US 1990-494024, filed on 15 Mar 1990, now abandoned Continuation-in-part of Ser. No. US 1989-449811, filed on 12 Dec 1989, now abandoned
DT Utility
FS Granted
LN.CNT 1480
INCL INCLM: 435/069.100
INCLS: 536/023.500; 435/320.100; 435/325.000; 435/352.000; 435/354.000; 435/357.000; 435/358.000; 435/364.000; 435/366.000; 435/367.000; 435/252.300; 435/252.330; 435/069.700; 435/069.800
NCL NCLM: 435/069.100
NCLS: 435/069.700; 435/069.800; 435/252.300; 435/252.330; 435/320.100; 435/325.000; 435/352.000; 435/354.000; 435/357.000; 435/358.000; 435/364.000; 435/366.000; 435/367.000; 536/023.500
IC [7]
ICM: C12N015-00
ICS: C12N005-02; C12P021-06; C07H021-04
EXF 435/6; 435/69.1; 435/69.8; 435/240.2; 435/252.3; 435/252.33; 435/320.1; 435/325; 435/352; 435/354; 435/357; 435/358; 435/364; 435/366; 435/367; 536/23.5; 536/24.3; 536/24.31; 536/24.33
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L4 ANSWER 12 OF 34 BIOSIS COPYRIGHT 2003 BIOLOGICAL ABSTRACTS INC.
AN 2001:562490 BIOSIS
DN PREV200100562490
TI A **naturally** occurring, high affinity p75 ligand which selectively activates p75, but not Trk receptors.
AU Lee, R. (1); Kermani, P. (1); Salzer, J. L.; Hempstead, B. L. (1)
CS (1) Weill Med Cornell Univ, New York, NY USA
SO Society for Neuroscience Abstracts, (2001) Vol. 27, No. 2, pp. 1804. print.
Meeting Info.: 31st Annual Meeting of the Society for Neuroscience San Diego, California, USA November 10-15, 2001
ISSN: 0190-5295.
DT Conference
LA English
SL English

L4 ANSWER 13 OF 34 USPATFULL
AN 2000:31394 USPATFULL
TI Neurotrophic factor (NT 4)
IN Rosenthal, Arnon, Pacifica, CA, United States
PA Genentech, Inc., South San Francisco, CA, United States (U.S. corporation)
PI US 6037320 20000314
AI US 1997-928694 19970912 (8)
RLI Continuation of Ser. No. US 1995-451947, filed on 26 May 1995, now patented, Pat. No. US 5702906 which is a division of Ser. No. US 1995-426419, filed on 19 Apr 1995, now abandoned which is a continuation

ICS: C07K014-475
EXF 514/2; 514/12; 530/350
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L4 ANSWER 14 OF 34 PCTFULL COPYRIGHT 2003 Univentio
AN 2000026348 PCTFULL ED 20020515
TIEN MAMMALIAN SUBTILISIN/KEXIN ISOZYME SKI-1: A PROPROTEIN CONVERTASE WITH A UNIQUE CLEAVAGE SPECIFICITY
TIFR SUBTILISINE/KEXINE ISOZYME SKI-1 MAMMIFERE : PROPROTEINE CONVERTASE DOTEE D'UNE SPECIFICITE DE CLIVAGE UNIQUE
IN SEIDAH, Nabil;
CHRETIEN, Michel;
MARCINKIEWICZ, Mieczyslaw;
LAAKSONEN, Reijo;
DAVIGNON, Jean
PA INSTITUT DE RECHERCHES CLINIQUES DE MONTREAL;
SEIDAH, Nabil;
CHRETIEN, Michel;
MARCINKIEWICZ, Mieczyslaw;
LAAKSONEN, Reijo;
DAVIGNON, Jean
LA English
DT Patent
PI WO 2000026348 A2 20000511
DS W: AE AL AM AT AU AZ BA BB BG BR BY CA CH CN CR CU CZ DE DK DM
EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ
LC LK LR LS LT LU LV MA MD MG MK MN MW MX NO NZ PL PT RO RU
SD SE SG SI SK SL TJ TM TR TT TZ UA UG US UZ VN YU ZA ZW GH
GM KE LS MW SD SL SZ TZ UG ZW AM AZ BY KG KZ MD RU TJ TM AT
BE CH CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE BF BJ CF
CG CI CM GA GN GW ML MR NE SN TD TG
AI WO 1999-CA1058 A 19991104
PRAI CA 1998-2,249,648 19981104
ICM C12N009-64
ICS C07K014-81

L4 ANSWER 15 OF 34 PCTFULL COPYRIGHT 2003 Univentio
AN 2000022119 PCTFULL ED 20020515
TIEN METHOD FOR OBTAINING ACTIVE β-NGF
TIFR PROCEDE D'OBTENTION DE NGF-β ACTIF
IN RUDOLPH, Rainer;
RATTENHOLL, Anke;
SCHWARZ, Elisabeth;
GROSSMANN, Adelbert
PA RUDOLPH, Rainer;
RATTENHOLL, Anke;
SCHWARZ, Elisabeth;
GROSSMANN, Adelbert
LA German
DT Patent
PI WO 2000022119 A1 20000420
DS W: AU BR CA JP KR US ZA
AI WO 1999-EP7613 A 19991011
PRAI EP 1998-98119077.0 19981009
ICM C12N015-12
ICS C07K014-48

L4 ANSWER 16 OF 34 USPATFULL
AN 1999:167121 USPATFULL
TI [illegible]

[illegible]
PI US 6005081 19991221
AI US 1997-970869 19971114 8

DT Utility
FS Granted
LN.CNT 2397
INCL INCLM: 530/399.000
INCLS: 530/324.000; 530/350.000; 530/412.000; 530/416.000; 530/417.000; 435/069.100; 435/069.400; 435/070.100; 435/071.100
NCL NCLM: 530/399.000
NCLS: 435/069.100; 435/069.400; 435/070.100; 435/071.100; 530/324.000; 530/350.000; 530/412.000; 530/416.000; 530/417.000
IC [6]
ICM: C07K003-14
ICS: C12P021-06
EXF 530/399; 530/324; 530/350; 530/412; 530/416; 530/417; 435/69.1; 435/69.4; 435/70.1; 435/71.1
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L4 ANSWER 17 OF 34 USPATFULL
AN 1999:137455 USPATFULL
TI Transferrin receptor specific ligand-neuropharmaceutical agent fusion proteins
IN Friden, Phillip M., Bedford, MA, United States
Starzyk, Ruth M., Framingham, MA, United States
Morrison, Sherie L., Los Angeles, CA, United States
Park, Eun-Chung, Boston, MA, United States
McGrath, John P., Cambridge, MA, United States
PA Alkermes, Inc., United States (U.S. corporation)
The Regents of the University of California, United States (U.S. corporation)
PI US 5977307 19991102
WO 9521245 19950810
AI US 1996-581543 19960213 (8)
WO 1995-US1469 19950203
19961126 PCT 371 date
19961126 PCT 102(e) date
RLI Continuation-in-part of Ser. No. US 1993-94534, filed on 16 Jul 1993, now patented, Pat. No. US 5672683 which is a continuation-in-part of Ser. No. US 1992-999803, filed on 20 Nov 1992, now abandoned which is a division of Ser. No. US 1992-846830, filed on 6 Mar 1992, now patented, Pat. No. US 5182107 which is a continuation-in-part of Ser. No. WO 1990-US5077, filed on 7 Sep 1990 which is a continuation-in-part of Ser. No. US 1989-404089, filed on 7 Sep 1989, now patented, Pat. No. US 5154924
DT Utility
FS Granted
LN.CNT 2264
INCL INCLM: 530/350.000
INCLS: 530/387.100; 530/399.000; 435/069.700; 536/023.400
NCL NCLM: 530/350.000
NCLS: 435/069.700; 530/387.100; 530/399.000; 536/023.400
IC [6]
ICM: C07K001-00
ICS: C07K014-00, A61K038-24; C07H021-04
EXF 530/350; 530/387.4; 530/399; 530/324; 530/387.1; 435/69.7; 536/23.4
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L4 ANSWER 18 OF 34 USPATFULL
AN 1999:92541 USPATFULL
TI Protein expression system
IN Sgarlato, Gregory D., Los Gatos, CA, United States
PA Technologene, Inc., Los Gatos, CA, United States

INCL INCLM: 435/069.1
INCLS: 435/069.8[illegible]
NCL NCLM: 435/069.7[illegible]

IC [6]
ICM: C07K019-00
ICS: C12N015-62
EXF 435/69.7; 435/69.8; 435/207; 435/68.1; 436/532; 436/828; 530/387.1; 530/350; 530/413; 530/812; 530/866; 530/867; 536/23.4; 536/23.2; 536/23.53; 536/23.7; 935/47
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L4 ANSWER 19 OF 34 USPATFULL
AN 1998:135007 USPATFULL
TI Neurotrophic factor
IN Rosenthal, Arnon, Pacifica, CA, United States
PA Genentech, Inc., South San Francisco, CA, United States (U.S. corporation)
PI US 5830858 19981103
AI US 1995-424826 19950419 (8)
RLI Continuation of Ser. No. US 1994-240387, filed on 10 May 1994, now abandoned which is a continuation of Ser. No. US 1991-648482, filed on 13 Jan 1991, now abandoned which is a continuation-in-part of Ser. No. US 1990-587707, filed on 25 Sep 1990, now patented, Pat. No. US 5364769
DT Utility
FS Granted
LN.CNT 2363
INCL INCLM: 514/012.000
INCLS. 514/002.000, 530/350.000, 530/395.000, 530/399.000, 530/402.000; 435/069.100
NCL NCLM: 514/012.000
NCLS: 435/069.100; 514/002.000; 530/350.000; 530/395.000; 530/399.000; 530/402.000
IC [6]
ICM: A61K038-18
ICS: C07K014-475
EXF 514/2; 514/12; 530/350; 530/395; 530/399; 530/402; 435/69.1
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L4 ANSWER 20 OF 34 USPATFULL
AN 1998:1897 USPATFULL
TI Neurotrophic factors having altered receptor binding specificities
IN Persson, Hakan Bengt, Vreta Gard, S-14743 Tumba, Sweden
Moliner, Carlos Fernando Ibanez, Tangvagen 29, S-12638 Hagersten, Sweden
PI US 5705617 19980106
AI US 1994-300044 19940902 (8)
RLI Division of Ser. No. US 1992-847369, filed on 6 Mar 1992, now patented, Pat. No. US 5349055
DT Utility
FS Granted
LN.CNT 1195
INCL INCLM: 530/399.000
INCLS: 530/350.000
NCL NCLM: 530/399.000
NCLS: 530/350.000
IC [6]
ICM: C07K014-475
EXF 530/399; 514/12; 435/69.4; 435/320.1
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L4 ANSWER 21 OF 34 USPATFULL
AN 97:123048 USPATFULL
TI Antibodies tc neurotrophic factor 4 (NT-4)
IN Rosenthal, Arnon, Pacifica, CA, United States

Continuation of Ser. No. US 1991-648482, filed on 13 Jan 1991, now abandoned which is a continuation-in-part of Ser. No. US 1990-587707,

5364769
DT Utility
FS Granted
LN.CNT 2046
INCL INCLM: 435/007.100
INCLS: 530/387.100; 530/387.900; 530/388.240; 530/413.000; 435/336.000; 435/236.000
NCL NCLM: 435/007.100
NCLS: 435/236.000; 435/336.000; 530/387.100; 530/387.900; 530/388.240; 530/413.000
IC [6]
ICM: G01N033-53
ICS: C12N005-12; C07K016-22; C07K001-16
EXF 424/139.1; 424/141.1; 424/145.1; 424/9.1; 435/7.1; 435/336; 530/387.1; 530/387.9; 530/388.1; 530/388.15; 530/388.24; 530/389.1; 530/389.2; 530/391.3; 530/413
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L4 ANSWER 22 OF 34 PCTFULL COPYRIGHT 2003 Univentio
AN 1997028272 PCTFULL ED 20020514
TIEN PROTEIN EXPRESSION SYSTEM
TIFR SYSTEME D'EXPRESSION DE PROTEINES
IN SGARLATO, Gregory, D.
PA TECHNOLOGENE INC.
LA English
DT Patent
PI WO 9728272 A1 19970807
DS W: AU CA JP AT BE CH DE DK ES FI FR GB GR IE IT LU MC NL PT SE
AI WO 1997-US1470 A 19970131
PRAI US 1996-8/595,043 19960131
ICM C12P021-00
ICS C12P021-06; C07K001-12; C07K001-22; C07K014-46; C07K014-195; C07K016-00; C07K019-00; C07H021-04; C12N009-38

L4 ANSWER 23 OF 34 MEDLINE DUPLICATE 1
AN 96177872 MEDLINE
DN 96177872 PubMed ID: 8615794
TI Cellular processing of the nerve growth factor precursor by the mammalian pro-protein convertases.
AU Seidah N G; Benjannet S; Pareek S; Savaria D; Hamelin J; Goulet B; Laliberte J; Lazure C; Chretien M; Murphy R A
CS J. A. DeSeve Laboratories of Biochemical and Molecular Neuroendocrinology, Clinical Research Institute of Montreal, University of Montreal, Canada.
SO BIOCHEMICAL JOURNAL, (1996 Mar 15) 314 (Pt 3) 951-60.
Journal code: 2984726R. ISSN: 0264-6021.
CY ENGLAND: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199606
ED Entered STN: 19960613
Last Updated on STN: 20000303
Entered Medline: 19960603

L4 ANSWER 24 OF 34 PCTFULL COPYRIGHT 2003 Univentio
AN 1995002421 PCTFULL ED 20020514
TIEN TRANSFERRIN RECEPTOR SPECIFIC LIGAND NEUROPHARMACEUTICAL AGENT FUSION PROTEINS
TIFR PROTEINES DE FUSION A LIGAND SPECIFIQUE DU RECEPTEUR DE LA TRANSFERRINE ET A AGENT NEUROPHARMACEUTIQUE
[illegible]
PA ALKERMES, INC.;
THE REGENTS OF THE UNIVERSITY OF CALIFORNIA;
[illegible]

MORRISON, Sherie, L.;
PARK, Eun-Chung;
McGRATH, John, P.
LA English
DT Patent
PI WO 9502421 A1 19950126
DS W: AM AT AU BB BG BR BY CA CH CN CZ DE DK ES FI GB GE HU JP KE
KG KP KR KZ LK LT LU LV MD MG MN MW NL NO NZ PL PT RO RU SD
SE SI SK TJ TT UA US UZ VN KE MW SD AT BE CH DE DK ES FR GB
GR IE IT LU MC NL PT SE BF BJ CF CG CI CM GA GN ML MR NE SN
TD TG
AI WO 1994-US8000 A 19940718
PRAI US 1993-8/094,534 19930716
ICM A61K047-48
ICS C07K014-65

L4 ANSWER 25 OF 34 USPATFULL
AN 94:99824 USPATFULL
TI Nucleic acid encoding neurotrophic factor four (NT-4), vectors, host cells and methods of production
IN Rosenthal, Arnon, Pacifica, CA, United States
PA Genentech, Inc., South San Francisco, CA, United States (U.S. corporation)
PI US 5364769 19941115
AI US 1990 587707 19900925 (7)
DT Utility
FS Granted
LN.CNT 1357
INCL INCLM: 435/069.100
INCLS: 435/069.400; 435/320.100; 435/240.100; 435/240.200; 536/023.500; 536/023.510
NCL NCLM: 435/069.100
NCLS: 435/069.400; 435/320.100; 435/369.000; 536/023.500; 536/023.510
IC [5]
ICM: C12N005-10
ICS: C12N015-18; C12N015-12
EXF 536/27; 536/23.50; 536/23.51; 536/252.3; 435/69.1; 435/69.4; 435/320.1; 435/240.1; 435/240.2
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L4 ANSWER 26 OF 34 USPATFULL
AN 94:82349 USPATFULL
TI Nerve growth factor having altered receptor binding specificities
IN Persson, Hakan B., Vreta Gard, S-14743 Tumba, Sweden
Moliner, Carlos F. I., Tangvagen 29, S-12638 Hagersten, Sweden
PI US 5349055 19940920
AI US 1992 847369 19920306 (7)
DT Utility
FS Granted
LN.CNT 1154
INCL INCLM: 530/399.000
INCLS: 930/120.000
NCL NCLM: 530/399.000
NCLS: 930/120.000
IC [5]
ICM: C07K013-00
EXF 530/399; 530/387; 930/120
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L4 ANSWER 27 OF 34 [illegible]

[illegible]
IN FAHN, Axel;
MALLET, Jacques;
[illegible]

ROBERT, Jean-Jacques;
LE GAL LA SALLE, Gildas
PA RHONE-POULENC RORER S.A.;
INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE;
KAHN, Axel;
MALLET, Jacques;
PERRICAUDET, Michel;
PESCHANSKI, Marc;
ROBERT, Jean-Jacques;
LE GAL LA SALLE, Gildas
LA French
DT Patent
PI WO 9408026 A1 19940414
DS W: AU CA FI HU JP NO NZ US AT BE CH DE DK ES FR GB GR IE IT LU
MC NL PT SE
AI WO 1993-EP2519 A 19930917
PRAI FR 1992-92402644.6 19920925
ICM C12N015-86
ICS C12N015-00; A61K039-235; C12N015-11; C12N005-10; A61K048-00

L4 ANSWER 28 OF 34 PCTFULL COPYRIGHT 2003 Univentio
AN 1993025684 PCTFULL ED 20020513
TIEN THERAPEUTIC AND DIAGNOSTIC METHODS BASED ON NEUROTROPHIN-4 EXPRESSION
TIFR PROCEDES THERAPEUTIQUE ET DIAGNOSTIQUE BASES SUR L'EXPRESSION DE LA NEUROTROPHINE-4
IN IP, Nancy;
ALTAR, Charles, A.;
DISTEFANO, Peter;
VENTIMIGLIA, Roseann;
WIEGAND, Stanley;
WONG, Vivien;
YANCOPOULOS, George, D.
PA REGENERON PHARMACEUTICALS, INC.;
IP, Nancy;
ALTAR, Charles, A.;
DISTEFANO, Peter;
VENTIMIGLIA, Roseann;
WIEGAND, Stanley;
WONG, Vivien;
YANCOPOULOS, George, D.
LA English
DT Patent
PI WO 9325684 A1 19931223
DS W: AU BB BG BR BY CA CZ FI HU JP KR KZ LK MG MN MW NO NZ PL RO
RU SD SK UA US AT BE CH DE DK ES FR GB GR IE IT LU MC NL PT
SE BF BJ CF CG CI CM GA GN ML MR NE SN TD TG
AI WO 1993-US5672 A 19930611
PRAI US 1992-898,194 19920612
ICM C12N015-12
ICS C12Q001-68; C12P021-08; A61K037-02

L4 ANSWER 29 OF 34 PCTFULL COPYRIGHT 2003 Univentio
AN 1993018066 PCTFULL ED 20020513
TIEN NEUROTROPHIC FACTORS HAVING ALTERED RECEPTOR BINDING SPECIFICITIES
TIFR FACTEURS NEUROTROPHIQUES DOTES DE PROPRIETES MODIFIEES CONCERNANT LA LIAISON A DES RECEPTEURS
IN PERSSON, Hakan, Bengt;
MOLINER, Carlos, Fernando, Ibanez
PA PERSSON, Hakan, Bengt;
MOLINER, Carlos, Fernando, Ibanez

[illegible]
BF BJ CF CG CI CM GA GN ML MR NE SN TD TG
AI WO 1993-[illegible] A 19930[illegible]
PRAI [illegible] [illegible]

L4 ANSWER 30 OF 34 PCTFULL COPYRIGHT 2003 Univentio
AN 1993010150 PCTFULL ED 20020513
TIEN EXPRESSION OF NEUROTROPHIC FACTORS WITH HETEROLOGOUS PREPRO REGIONS
TIFR EXPRESSION DE FACTEURS NEUROTROPHIQUES AU MOYEN DE REGIONS PREPRO HETEROLOGUES
IN SQUINTO, Stephen, P.;
IP, Nancy;
GIES, David;
YANCOPOULOS, George, D.;
HU, Shaw-Fen, Sylvia
PA REGENERON PHARMACEUTICALS, INC.;
AMGEN, INC.
LA English
DT Patent
PI WO 9310150 A1 19930527
DS W: AU BB BG BR CA CS FI HU JP KR LK MG MN MW NO PL RO RU SD UA
AT BE CH DE DK ES FR GB GR IE IT LU MC NL SE BF BJ CF CG CI
CM GA GN ML MR SN TD TG
AI WO 1992-US9792 A 19921113
PRAI US 1991-792,492 19911114
ICM C07K013-00
ICS C12N015-18; C12N001-21; C12N015-67

L4 ANSWER 31 OF 34 PCTFULL COPYRIGHT 2003 Univentio
AN 1992020365 PCTFULL ED 20020513
TIEN THERAPEUTIC AND DIAGNOSTIC METHODS BASED ON NEUROTROPHIN-4 EXPRESSION
TIFR PROCEDES THERAPEUTIQUES ET DIAGNOSTIQUES BASEES SUR L'EXPRESSION DE NEUROTROPHINE-4
IN HALLBOOK, Finn;
IBANEZ MOLINER, Carlos, Fernando;
PERSSON, Hakan, Bengt;
IP, Nancy;
YANCOPOULOS, George, D.
PA REGENERON PHARMACEUTICALS, INC.;
HALLBOOK, Finn;
IBANEZ MOLINER, Carlos, Fernando;
PERSSON, Hakan, Bengt
LA English
DT Patent
PI WO 9220365 A1 19921126
DS W: AT AU BE CA CH CS DE DK ES FI FR GB GR HU IT JP KR LU MC NL
NO RU SE
AI WO 1992-US4266 A 19920520
PRAI US 1991-703,450 19910521
US 1991-729,253 19910712
US 1991-734,422 19910723
US 1991-751,356 19910828
US 1991-762,674 19910920
US 1991-791,924 19911114
ICM A61K037-02
ICS A61K049-00; G01N033-50; G01N033-68; C07K039-00; C12N015-12; C12N015-79

L4 ANSWER 32 OF 34 PCTFULL COPYRIGHT 2003 Univentio
AN 1992005254 PCTFULL ED 20020513
TIEN NOVEL NEUROTHROPHIC FACTOR
TIFR NOUVEAU FACTEUR NEUROTROPHIQUE
IN ROSENTHAL, Arnon
PA GENENTECH, INC.;

[illegible]
AI WO 1991-US6402 A 19910904
PRAI US 1990-587,715 19900925
US 1991-[illegible] [illegible]

ICS C12Q001-68; C12P021-08; [redacted]K037-02

L4 ANSWER 33 OF 34 PCTFULL COPYRIGHT 2003 Univentio
AN 1990013650 PCTFULL ED 20020513
TIEN A PLASMID DNA CONSTRUCT INCLUDING THE GENE ENCODING A MAMMALIAN BETA-NERVE GROWTH FACTOR
TIFR CONSTRUCTION D'ADN PLASMIDAL COMPRENANT LE GENE CODANT UN FACTEUR DE CROISSANCE BETA-NERVEUX CHEZ LES MAMMIFERES
IN OLSON, Lars;
PERSSON, Hakan;
EBENDAL, Ted
PA LOPE MEDICINE AB;
OLSON, Lars;
PERSSON, Hakan;
EBENDAL, Ted
LA English
DT Patent
PI WO 9013650 A1 19901115
DS W: AT AU BE CA CH DE DK ES FI FR GB IT JP LU NL NO SE US
AI WO 1990-SE301 A 19900508
PRAI SE 1989-8901715-6 19890512
ICM C12N015-18
ICS C12N015-85

L4 ANSWER 34 OF 34 MEDLINE DUPLICATE 2
AN 89178770 MEDLINE
DN 89178770 PubMed ID: 2648014
TI Synthesis of chimeric mouse nerve growth factor precursor and human beta-nerve growth factor in Escherichia coli: immunological properties.
AU Dicou E; Houlgatte R; Lee J; von Wilcken-Bergmann B
CS INSERM U, Centre Hospitalier Regional, Angers, France.
SO JOURNAL OF NEUROSCIENCE RESEARCH, (1989 Jan) 22 (1) 13-9.
Journal code: 7600111. ISSN: 0360-4012.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 198904
ED Entered STN: 19900306
Last Updated on STN: 19900306
Entered Medline: 19890427

=> s l4 and arginine
L5 22 L4 AND ARGININE

=> d 1-22

L5 ANSWER 1 OF 22 USPATFULL
AN 2002:251935 USPATFULL
TI Purification of NGF
IN Burton, Louis E., San Mateo, CA, UNITED STATES
Schmelzer, Charles H., Burlingame, CA, UNITED STATES
Beck, Joanne T., Westlake Village, CA, UNITED STATES
PI US 2002137893 A1 20020926
AI US 2002-72681 A1 20020208 (10)
RLI Continuation of Ser. No. US 2000-675503, filed on 29 Sep 2000, GRANTED, Pat. No. US 6423831 Continuation of Ser. No. US 1999 363573, filed on 29 Jul 1999, GRANTED, Pat. No. US 6184360 Continuation of Ser. No. US

[redacted]
INCLM: [redacted]
INCLS: [redacted]

IC [7]
ICM: C07K014-435
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L5 ANSWER 2 OF 22 USPATFULL
AN 2002:181791 USPATFULL
TI Isolation of neurotrophins from a mixture containing other proteins and neurotrophin variants using hydrophobic interaction chromatography
IN Burton, Louis E., San Mateo, CA, United States
Schmelzer, Charles H., Burlingame, CA, United States
Beck, Joanne T., Westlake Village, CA, United States
PA Genentech, Inc., So. San Francisco, CA, United States (U.S. corporation)
PI US 6423831 B1 20020723
AI US 2000-675503 20000929 (9)
RLI Continuation of Ser. No. US 1999-363573, filed on 29 Jul 1999, now patented, Pat. No. US 6184360 Continuation of Ser. No. US 1997-970865, filed on 14 Nov 1997, now patented, Pat. No. US 6005081
PRAI US 1997-47855P 19970529 (60)
US 1996-30838P 19961115 (60)
DT Utility
FS GRANTED
LN.CNT 2348
INCL INCLM: 530/399.000
INCLS: 530/324.000; 530/350.000; 530/412.000; 530/416.000; 530/417.000; 435/069.100; 435/069.400; 435/070.100; 435/071.100
NCL NCLM: 530/399.000
NCLS: 435/069.100; 435/069.400; 435/070.100; 435/071.100; 530/324.000; 530/350.000; 530/412.000; 530/416.000; 530/417.000
IC [7]
ICM: C07K003-14
ICS: C12P021-06
EXF 530/399; 530/324; 530/350; 530/412; 530/416; 530/417; 435/69.1; 435/69.4; 435/70.1; 435/71.1
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L5 ANSWER 3 OF 22 USPATFULL
AN 2002:85534 USPATFULL
TI NOVEL NEUROTROPHIC FACTOR
IN ROSENTHAL, ARNON, PACIFICA, CA, UNITED STATES
PI US 2002045576 A1 20020418
US 6506728 B2 20030114
AI US 1995-450842 A1 19950526 (8)
RLI Division of Ser. No. US 1995-426419, filed on 19 Apr 1995, ABANDONED Continuation of Ser. No. US 1993-30013, filed on 22 Mar 1993, ABANDONED A 371 of International Ser. No. WO 1991-US6950, filed on 24 Sep 1991, UNKNOWN Continuation-in-part of Ser. No. US 1991-648482, filed on 31 Jan 1991, ABANDONED Continuation-in-part of Ser. No. US 1990-587707, filed on 25 Sep 1990, GRANTED, Pat. No. US 5364769
DT Utility
FS APPLICATION
LN.CNT 2815
INCL INCLM: 514/012.000
INCLS: 514/002.000
NCL NCLM: 514/012.000
NCLS: 514/002.000
IC [7]
ICM: A01N037-16
ICS: A61K038-17
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

[illegible]
Beck, Joanne T., Westlake Village, CA, United States
PA Genentech, Inc., South San Francisco, CA, United States (U.S.
[illegible]

AI US 1999-363573 [redacted]990729 (9)
RLI Continuation of Ser. No. US 1997-970865, filed on 14 Nov 1997, now patented, Pat. No. US 6005081
PRAI US 1996-30838P 19961115 (60)
US 1997-47855P 19970529 (60)
DT Utility
FS Granted
LN.CNT 2226
INCL INCLM: 530/399.000
INCLS: 530/324.000; 530/350.000; 530/412.000; 530/416.000; 530/417.000; 435/069.100; 435/069.400; 435/070.100; 435/071.100
NCL NCLM: 530/399.000
NCLS: 435/069.100; 435/069.400; 435/070.100; 435/071.100; 530/324.000; 530/350.000; 530/412.000; 530/416.000; 530/417.000
IC [7]
ICM: C07K003-14
ICS: C12P021-06
EXF 530/399; 530/324; 530/350; 530/412; 530/416; 530/417; 435/69.1; 435/69.4; 435/70.1; 435/71.1
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L5 ANSWER 5 OF 22 USPATFULL
AN 2001:7868 USPATFULL
TI Neuronal factor
IN Rosenthal, Arnon, Pacifica, CA, United States
Winslow, John W., El Granada, CA, United States
PA Genentech, Inc., So. San Francisco, CA, United States (U.S. corporation)
PI US 6174701 B1 20010116
AI US 1995-455741 19950531 (8)
RLI Continuation of Ser. No. US 1995-381030, filed on 31 Jan 1995
Continuation of Ser. No. US 1990-494024, filed on 15 Mar 1990, now abandoned Continuation-in-part of Ser. No. US 1989-449811, filed on 12 Dec 1989, now abandoned
DT Utility
FS Granted
LN.CNT 1480
INCL INCLM: 435/069.100
INCLS: 536/023.500; 435/320.100; 435/325.000; 435/352.000; 435/354.000; 435/357.000; 435/358.000; 435/364.000; 435/366.000; 435/367.000; 435/252.300; 435/252.330; 435/069.700; 435/069.800
NCL NCLM: 435/069.100
NCLS: 435/069.700; 435/069.800; 435/252.300; 435/252.330; 435/320.100; 435/325.000; 435/352.000; 435/354.000; 435/357.000; 435/358.000; 435/364.000; 435/366.000; 435/367.000; 536/023.500
IC [7]
ICM: C12N015-00
ICS: C12N005-02; C12P021-06; C07H021-04
EXF 435/6; 435/69.1; 435/69.8; 435/240.2; 435/252.3; 435/252.33; 435/320.1; 435/325; 435/352; 435/354; 435/357; 435/358; 435/364; 435/366; 435/367; 536/23.5; 536/24.3; 536/24.31; 536/24.33
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L5 ANSWER 6 OF 22 USPATFULL
AN 2000:31394 USPATFULL
TI Neurotrophic factor (NT-4)
IN Rosenthal, Arnon, Pacifica, CA, United States
PA Genentech, Inc., South San Francisco, CA, United States (U.S. corporation)
PI US 6037320 20000314
[illegible]

[illegible]
FS Granted
LN.CNT 2746
[illegible]

NCL NCLM: 514/002.000
NCLS: 514/012.000; 530/[illegible].000
IC [7]
ICM: A61K038-18
ICS: C07K014-475
EXF 514/2; 514/12; 530/350
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L5 ANSWER 7 OF 22 USPATFULL
AN 1999:167121 USPATFULL
TI Purification of recombinant human neurotrophins
IN Burton, Louis E., San Mateo, CA, United States
Schmelzer, Charles H., Burlingame, CA, United States
Beck, Joanne T., Westlake Village, CA, United States
PA Genentech, Inc., South San Francisco, CA, United States (U.S. corporation)
PI US 6005081 19991221
AI US 1997-970865 19971114 (8)
PRAI US 1996-30838P 19961115 (60)
US 1997-47855P 19970529 (60)
DT Utility
FS Granted
LN.CNT 2397
INCL INCLM: 530/399.000
INCLS: 530/324.000; 530/350.000; 530/412.000; 530/416.000; 530/417.000; 435/069.100; 435/069.400; 435/070.100; 435/071.100
NCL NCLM: 530/399.000
NCLS: 435/069.100; 435/069.400; 435/070.100; 435/071.100; 530/324.000; 530/350.000; 530/412.000; 530/416.000; 530/417.000
IC [6]
ICM: C07K003-14
ICS: C12P021-06
EXF 530/399; 530/324; 530/350; 530/412; 530/416; 530/417; 435/69.1; 435/69.4; 435/70.1; 435/71.1
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L5 ANSWER 8 OF 22 USPATFULL
AN 1999:137455 USPATFULL
TI Transferrin receptor specific ligand-neuropharmaceutical agent fusion proteins
IN Friden, Phillip M., Bedford, MA, United States
Starzyk, Ruth M., Framingham, MA, United States
Morrison, Sherie L., Los Angeles, CA, United States
Park, Eun-Chung, Boston, MA, United States
McGrath, John P., Cambridge, MA, United States
PA Alkermes, Inc., United States (U.S. corporation)
The Regents of the University of California, United States (U.S. corporation)
PI US 5977307 19991102
WO 9521245 19950810
AI US 1996-581543 19960213 (8)
WO 1995-US1469 19950203
19961126 PCT 371 date
19961126 PCT 102(e) date
RLI Continuation-in-part of Ser. No. US 1993-94534, filed on 16 Jul 1993, now patented, Pat. No. US 5672683 which is a continuation-in-part of Ser. No. US 1992-999803, filed on 20 Nov 1992, now abandoned which is a division of Ser. No. US 1992 846830, filed on 6 Mar 1992, now patented, Pat. No. US 5182107 which is a continuation-in part of Ser. No. WO [illegible]

[illegible]
INCL INCLM: [illegible]
INCLS: [illegible]

IC [6]
ICM: C07K001-00
ICS: C07K014-00; A61K038-24; C07H021-04
EXF 530/350; 530/387.4; 530/399; 530/324; 530/387.1; 435/69.7; 536/23.4
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L5 ANSWER 9 OF 22 USPATFULL
AN 1999:92541 USPATFULL
TI Protein expression system
IN Sgarlato, Gregory D., Los Gatos, CA, United States
PA Technologene, Inc., Los Gatos, CA, United States (U.S. corporation)
PI US 5935824 19990810
AI US 1996-595043 19960131 (8)
DT Utility
FS Granted
LN.CNT 5959
INCL INCLM: 435/069.700
INCLS: 435/069.800; 530/350.000; 536/023.400
NCL NCLM: 435/069.700
NCLS: 435/069.800; 530/350.000; 536/023.400
IC [6]
ICM: C07K019-00
ICS: C12N015-62
EXF 435/69.7; 435/69.8; 435/207; 435/68.1; 436/532; 436/828; 530/387.1; 530/350; 530/413; 530/812; 530/866, 530/867, 536/23.4; 536/23.2; 536/23.53; 536/23.7; 935/47
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L5 ANSWER 10 OF 22 USPATFULL
AN 1998:135007 USPATFULL
TI Neurotrophic factor
IN Rosenthal, Arnon, Pacifica, CA, United States
PA Genentech, Inc., South San Francisco, CA, United States (U.S. corporation)
PI US 5830858 19981103
AI US 1995-424826 19950419 (8)
RLI Continuation of Ser. No. US 1994-240387, filed on 10 May 1994, now abandoned which is a continuation of Ser. No. US 1991-648482, filed on 13 Jan 1991, now abandoned which is a continuation-in-part of Ser. No. US 1990-587707, filed on 25 Sep 1990, now patented, Pat. No. US 5364769
DT Utility
FS Granted
LN.CNT 2363
INCL INCLM: 514/012.000
INCLS: 514/002.000; 530/350.000; 530/395.000; 530/399.000; 530/402.000; 435/069.100
NCL NCLM: 514/012.000
NCLS: 435/069.100; 514/002.000; 530/350.000; 530/395.000; 530/399.000; 530/402.000
IC [6]
ICM: A61K038-18
ICS: C07K014-475
EXF 514/2; 514/12; 530/350; 530/395; 530/399; 530/402; 435/69.1
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L5 ANSWER 11 OF 22 USPATFULL
AN 1998:1897 USPATFULL
TI Neurotrophic factors having altered receptor binding specificities
IN Persson, Hakan Bengt, Vreta Gard, S-14743 Tumba, Sweden
[illegible]

[illegible]
FS Granted
LN.CNT 1195
[illegible]

NCL NCLM: 530/399.000
NCLS: 530/350.000
IC [6]
ICM: C07K014-475
EXF 530/399; 514/12; 435/69.4; 435/320.1
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L5 ANSWER 12 OF 22 USPATFULL
AN 97:123048 USPATFULL
TI Antibodies to neurotrophic factor-4 (NT-4)
IN Rosenthal, Arnon, Pacifica, CA, United States
PA Genentech, Inc., South San Francisco, CA, United States (U.S. corporation)
PI US 5702906 19971230
AI US 1995-451947 19950526 (8)
RLI Division of Ser. No. US 1995-426419, filed on 19 Apr 1995 which is a continuation of Ser. No. US 1993-30013, filed on 22 Mar 1993, now abandoned which is a continuation-in-part of Ser. No. US 1991-648482, filed on 31 Jan 1991, now abandoned which is a continuation-in-part of Ser. No. US 1990-587707, filed on 25 Sep 1990, now patented, Pat. No. US 5364769
DT Utility
FS Granted
LN.CNT 2046
INCL INCLM: 435/007.100
INCLS: 530/387.100; 530/387.900; 530/388.240; 530/413.000; 435/336.000; 435/236.000
NCL NCLM: 435/007.100
NCLS: 435/236.000; 435/336.000; 530/387.100; 530/387.900; 530/388.240; 530/413.000
IC [6]
ICM: G01N033-53
ICS: C12N005-12; C07K016-22; C07K001-16
EXF 424/139.1; 424/141.1; 424/145.1; 424/9.1; 435/7.1; 435/336; 530/387.1; 530/387.9; 530/388.1; 530/388.15; 530/388.24; 530/389.1; 530/389.2; 530/391.3; 530/413
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L5 ANSWER 13 OF 22 USPATFULL
AN 94:99824 USPATFULL
TI Nucleic acid encoding neurotrophic factor four (NT-4), vectors, host cells and methods of production
IN Rosenthal, Arnon, Pacifica, CA, United States
PA Genentech, Inc., South San Francisco, CA, United States (U.S. corporation)
PI US 5364769 19941115
AI US 1990-587707 19900925 (7)
DT Utility
FS Granted
LN.CNT 1357
INCL INCLM: 435/069.100
INCLS: 435/069.400; 435/320.100; 435/240.100; 435/240.200; 536/023.500; 536/023.510
NCL NCLM: 435/069.100
NCLS: 435/069.400; 435/320.100; 435/369.000; 536/023.500; 536/023.510
IC [5]
ICM: C12N005-10
ICS: C12N015-18; C12N015-12
EXF 536/27; 536/23.50; 536/23.51; 536/252.3; 435/69.1; 435/69.4; 435/320.1;
[illegible]

[illegible]
IN Persson, Håkan B., Vinkla Gård, S-[illegible] Lund, Sweden
Mortimer, Carlos F. I., Tångvägen 8B, S-[illegible] Hägersten, Sweden
[illegible]

DT Utility
FS Granted
LN.CNT 1154
INCL INCLM: 530/399.000
INCLS: 930/120.000
NCL NCLM: 530/399.000
NCLS: 930/120.000
IC [5]
ICM: C07K013-00
EXF 530/399; 530/387; 930/120
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L5 ANSWER 15 OF 22 PCTFULL COPYRIGHT 2003 Univentio
AN 2002096356 PCTFULL ED 20021217 EW 200249
TIEN HIGH AFFINITY LIGAND FOR p75 NEUROTROPHIN RECEPTOR
TIFR LIGAND A FORTE AFFINITE POUR LE RECEPTEUR DE LA NEUROTROPHINE P75
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AG FEIT, Irving, N., Hoffmann & Baron, LLP, 6900 Jericho Turnpike, Syosset, NY 11791, US
LAF English
LA English
DT Patent
PI WO 2002096356 A2 20021205
DS W: AE AG AL AM AT AU AZ BA BB BG BR BY BZ CA CH CN CO CR CU CZ DE DK DM DZ EC EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX MZ NO NZ OM PH PL PT RO RU SD SE SG SI SK SL TJ TM TN TR TT TZ UA UG US UZ VN YU ZA ZM ZW
RW (ARIPO): GH GM KE LS MW MZ SD SL SZ TZ UG ZM ZW
RW (EAPO): AM AZ BY KG KZ MD RU TJ TM
RW (EPO): AT BE CH CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE TR
RW (OAPI): BF BJ CF CG CI CM GA GN GQ GW ML MR NE SN TD TG
AI WO 2002-US16540 A 20020524
PRAI US 2001-60/293,823 20010525
US 2001-60/305,510 20010713

L5 ANSWER 16 OF 22 PCTFULL COPYRIGHT 2003 Univentio
AN 1997028272 PCTFULL ED 20020514
TIEN PROTEIN EXPRESSION SYSTEM
TIFR SYSTEME D'EXPRESSION DE PROTEINES
IN SGARLATO, Gregory, D.
PA TECHNOLOGENE INC.
LA English
DT Patent

IC [illegible]
ICS C12P021 00; C07H001 10; C07K001 04; C07K014 47; C07K014 48; C07K016 00; C07K019 00; C07H021 04; C12N009 00

AN 1995002421 PCTFULL ED 20020514
TIEN TRANSFERRIN RECEPTOR SPECIFIC LIGAND-NEUROPHARMACEUTICAL AGENT FUSION PROTEINS
TIFR PROTEINES DE FUSION A LIGAND SPECIFIQUE DU RECEPTEUR DE LA TRANSFERRINE ET A AGENT NEUROPHARMACEUTIQUE
IN FRIDEN, Phillip, M.;
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MORRISON, Sherie, L.;
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McGRATH, John, P.
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STARZYK, Ruth, M.;
MORRISON, Sherie, L.;
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LA English
DT Patent
PI WO 9502421 A1 19950126
DS W: AM AT AU BB BG BR BY CA CH CN CZ DE DK ES FI GB GE HU JP KE KG KP KR KZ LK LT LU LV MD MG MN MW NL NO NZ PL PT RO RU SD SE SI SK TJ TT UA US UZ VN KE MW SD AT BE CH DE DK ES FR GB GR IE IT LU MC NL PT SE BF BJ CF CG CI CM GA GN ML MR NE SN TD TC
AI WO 1994-US8000 A 19940718
PRAI US 1993-8/094,534 19930716
ICM A61K047-48
ICS C07K014-65

L5 ANSWER 18 OF 22 PCTFULL COPYRIGHT 2003 Univentio
AN 1993025684 PCTFULL ED 20020513
TIEN THERAPEUTIC AND DIAGNOSTIC METHODS BASED ON NEUROTROPHIN-4 EXPRESSION
TIFR PROCEDES THERAPEUTIQUE ET DIAGNOSTIQUE BASES SUR L'EXPRESSION DE LA NEUROTROPHINE-4
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VENTIMIGLIA, Roseann;
WIEGAND, Stanley;
WONG, Vivien;
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DISTEFANO, Peter;
VENTIMIGLIA, Roseann;
WIEGAND, Stanley;
WONG, Vivien;
YANCOPOULOS, George, D.
LA English
DT Patent
PI WO 9325684 A1 19931223
DS W: AU BB BG BR BY CA CZ FI HU JP KR KZ LK MG MN MW NO NZ PL RO RU SD SK UA US AT BE CH DE DK ES FR GB GR IE IT LU MC NL PT SE BF BJ CF CG CI CM GA GN ML MR NE SN TD TG
AI WO 1993-US5672 A 19930611
PRAI US 1992-898,194 19920612
ICM C12N015-12
ICS [illegible]

[illegible]
LIAISON A DES RECEPTEURS
IN PERSSON, Hakan, Bengt;
[illegible]

MOLINER, Carlos, Fernando Ibanez
LA English
DT Patent
PI WO 9318066 A1 19930916
DS W: AU BB BG BR CA CZ FI HU JP KP KR KZ LK MG MN MW NO NZ PL RO
RU SD SK UA AT BE CH DE DK ES FR GB GR IE IT LU MC NL PT SE
BF BJ CF CG CI CM GA GN ML MR SN TD TG
AI WO 1993-SE201 A 19930308
PRAI US 1992-7/847,369 19920306
ICM C07K015-06

L5 ANSWER 20 OF 22 PCTFULL COPYRIGHT 2003 Univentio
AN 1993010150 PCTFULL ED 20020513
TIEN EXPRESSION OF NEUROTROPHIC FACTORS WITH HETEROLOGOUS PREPRO REGIONS
TIFR EXPRESSION DE FACTEURS NEUROTROPHIQUES AU MOYEN DE REGIONS PREPRO HETEROLOGUES
IN SQUINTO, Stephen, P.;
IP, Nancy;
GIES, David;
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HU, Shaw-Fen, Sylvia
PA REGENERON PHARMACEUTICALS, INC.;
AMGEN, INC.
LA English
DT Patent
PI WO 9310150 A1 19930527
DS W: AU BB BG BR CA CS FI HU JP KR LK MG MN MW NO PL RO RU SD UA
AT BE CH DE DK ES FR GB GR IE IT LU MC NL SE BF BJ CF CG CI
CM GA GN ML MR SN TD TG
AI WO 1992-US9792 A 19921113
PRAI US 1991-792,492 19911114
ICM C07K013-00
ICS C12N015-18; C12N001-21; C12N015-67

L5 ANSWER 21 OF 22 PCTFULL COPYRIGHT 2003 Univentio
AN 1992020365 PCTFULL ED 20020513
TIEN THERAPEUTIC AND DIAGNOSTIC METHODS BASED ON NEUROTROPHIN-4 EXPRESSION
TIFR PROCEDES THERAPEUTIQUES ET DIAGNOSTIQUES BASEES SUR L'EXPRESSION DE NEUROTROPHINE-4
IN HALLBOOK, Finn;
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PERSSON, Hakan, Bengt;
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IBANEZ MOLINER, Carlos, Fernando;
PERSSON, Hakan, Bengt
LA English
DT Patent
PI WO 9220365 A1 19921126
DS W: AT AU BE CA CH CS DE DK ES FI FR GB GR HU IT JP KR LU MC NL
NO RU SE
AI WO 1992-US4266 A 19920520
PRAI US 1991-703,450 19910521
US 1991-729,253 19910712
US 1991-734,422 19910723
US 1991-751,356 19910828
US 1991-762,674 19910920
US 1991-761,021 19911114

L5 ANSWER 22 OF 22 PCTFULL COPYRIGHT 2003 Univentio
AN 1992005254 PCTFULL ED 20020513
TIEN NOVEL NEUROTHROPHIC FACTOR
IN [illegible]

PA GENENTECH, INC.;
ROSENTHAL, Arnon
LA English
DT Patent
PI WO 9205254 A1 19920402
DS W: AT AU BE CA CH DE DK ES FR GB GR IT JP LU NL SE US
AI WO 1991-US6950 A 19910924
PRAI US 1990-587,707 19900925
US 1991-648,482 19910131
ICM C12N015-12
ICS C12Q001-68; C12P021-08; A61K037-02

L5 ANSWER 20 OF 22 PCTFUL COPYRIGHT 2003 Univentio

DETD Important neurotrophic factors identified to date include nerve growth factor (NGF; Levi-Montalcini and Angeletti, 1968, Phys, Rev. 48:534); neurotrophin-3 (NT-3; Hohn et al., 1990, **Nature** 344:339; Maisonpierre et al., 1990, Science 247:1446), brain-derived neurotrophic factor (BDNF; Barde et al., 1982, EMBO J, 10 1:549)r neuratrophin-4 (NT-4; Hallbook et al.., 1991, Neuron 6:845-858), and ciliary neurotrophic factor (CNTF; Lin et al., 1979, Science 246:1023), Neurotrophins are generally synthesized in vivo as preproll precursor proteins. The preproll 1 5 region ref ers to the NH2-terminus of the precursor which is proteolytically removed during biosynthesis of the mature, biologically active form of the protein. The pre region refers to the signal sequence normally removed-by proteolytic processing during translocation across the cell membrane to yield a pro-protein; the pro region is then removed by proteolytic, processing to yield the mature form (see e.cr,, Darnell et al,, 1990, Molecular Cell Biology 2d ed,, Scientific American Books, pp, 650-657)e 2, 1, 1, NERVE GROWTH FACTOR
Nerve growth factor (NGF) is by far the most fully characterized of these neurotrophic molecules and has been shown, both in vitro and in vivo, to be essential for the survival of sympathetic and neural crest-derived sensory neurons during early development of both chick and rat (Levi-Montalcini and Angeletti, 1963r Develop6 Biol, 7:653-659; Levi-Montalcini et al., 1968, Physiol, Rev, 48:524-569). Until recently, almost all studies of NGF had focused on its role in the peripheral nervous system, but it now appears that NGF also influences the development and maintenance of specific populations of neurons in the central nervous system (Thoenen et al., 1987, Rev. Physiol. Biochem.

Natl. Acad. Sci. -68:2417-2420). The NGF gene has now been cloned from many species, including mouse (Scott et al,, 1983, **Nature** 302:538-540, human (Uilrich et al,, 1983, **Nature** 303: 821-825), cow and chick (Meier 1 5et al., 1986, EMBO J, 5:1489-1493), and rat (Whittemore et al., 1988, J. Neurosci. Res., 20:402-410) using conventional molecular biology techniques based on the availability of the protein sequence of mouse NGF to design suitable oligonucleotide probes.

The mouse NGF gene encompasses approximately 45 kb, containing several small 51 exons, with alternating splicing resulting in four distinct mRNA species (Serby, et al,, 1987, Mol. Cell. Biol. 7:3057-3064). Two major transcripts result in a long and 25 short NGF prepropeptide (Edwards, et al,, 1986, **Nature** 319:784-787; Serby, et al,, 1987, Mol. Cell.

[illegible]

[illegible] **Nature** [illegible]
The translation, processing and secretion of the NGF

Harbor Symp. Quan, Biol, [redacted]:427-433). on the strength of the reported cDNA sequence encoding mouse NGF (Scott, et al., 1983, **Nature** 302: 538-540). utilized an in vitro cell free translation system to identify key intermediates in the biosynthesis of the 7S complex of NGF. The signal sequence of the prepro NGF precursor is removed via proteolytic processing to yield a pro-NGF species of approximately 31 kD, The pro-region of the pro-NGF intermediate contains a pair of **arginine** residues known to be endoproteolytic processing sites, Proteolytic processing at either of these residues results in an additional major (21 kD) and minor (18,.5 kD) intermediate species, The mature form of NGF can be proteolytically derived from either of the above-mentioned intermediate species, At some point in the biosynthesis of the mature form of NGF, a COOH-terminal dipeptide (arg-gly) is proteolytically released,
The 7-subunit has been shown in vivo to proteolytically cleave the pro-NGF precursor to the mature form of NGF (Edwards, et al., 1988, J, Biole Chem, 263: 6810-6815), Attempts to mimic the process in vitro were unsuccessful, resulting in unfaithful processing of the pro-NGF precursor, presumably due to aberrant folding of the in vitro translation product.

Silen and Agard (1989, **Nature** 341:462-464) demonstrated that the pro region may facilitate proper folding of the a-lytic protease precursor, Therefore, 5 the pro region of the NGF precursor may also be required for proper folding prior to endoproteolytic processing to the mature form and association into the biologically active 7S NGF complex. Support for this hypothesis is documented in Suter et al, (1991, EMBO 10 J, 10:2395-2400), who assigned functions for two partially conserved domains within the pro-region of NGF, Domain I was shown to be essential for NGF expression in COS cells. Additionally, Domain II, located in the NGF pro-region proximal to the mature 15 coding region, was found to be involved in proteolytic processing.

It was noted that the highly basic **nature** and molecular size of BDNF were very similar to the NGF monomer.

Two recent studies with BDNF (Kalcheim, et al., 1987, EMBO J. 6:2871-2873; Hofer and Barde, 1988, **Nature** 331:261-262) have, however, indicated a physiological role of BDNF in avian PNS development, In addition to its effect on peripheral sensory neurons of both neural crest and neural placode origin, BDNF has been found to support the survival of developing CNS neurons; Johnson et al, (1986, J.

[illegible] pI of about 9,5, was found to resemble that established for NGF and BDNF; a putative signal

be followed by a prosequence of 121 amino acids (as compared with a prosequence of 103 amino acids in mouse NGF and a prosequence of 112 amino acids in 15 mouse BDNF). A comparison between mature mouse NGF, BDNF, and NT-3 revealed 54 amino acid identities, All 6 cysteine residues, known in NGF and BDNF to be involved in the formation of disulfide bridges (Leibrock et alor 1989f **Nature** 341:149-152; Angeletti, 20 1973r Biochem, 12:100-115), are amongst the conserved residues. Similarly, mature rat NT-3 appears to share 57% amino acid homology with rat NGF, and 581 amino acid homology with rat BDNF; 57 of the 120 residues (48%) appear to be shared by all three proteins.

All using animal cell expression systems (mammalian kidney cells), Liebrock et al. [**Nature** 341:149 (1989)] reported the expression of biologically active pig BDNF, and Rosenthal et al, [Neuron 4: 767 (1990)], Maisonpierre et al, (Science 10 247:1446 (1990)] and Hohn et al, [**Nature** 344:339 (1990)] separately reported the expression of biologically active NT-3 of various species. In additionr Chan et al, CEP Publication No. 370171, published May 1990) reported the expression of biologically active mature human BDNF from insect cells by way of a baculovirus expression system, Regarding microbial production of neurotrophins, Iwai et al. [Chem, Pharm. Bull, 34:4727 (1986)] reported the expression of synthetic genes for human NGF and a fusion thereof in E. coli, The product was only characterized by molecular weight, after treatment with a reducing agent, and there was no information regarding the presence of biological activity.

5,1 THE EXPRESSION PRODUCTS OF THE PRESENT INVENTION

I 0

The bioactive proteins which can be obtained according to the present invention are the mature neurotrophic factors which are members of the neurotrophin gene family, or biologically active portions or derivatives thereof. The term biologically active as used herein refers to the ability to express one or more biological activities of the full-length mature neurotrophin. Such neurotrophins include but are not limited to mature BDNF, NT NGF and NT-4 and such other members as are identified by those methods utilized to determine members of the neurotrophin gene family (e.g., using molecular probes, generated by PCR, corresponding to regions of homology within' the family; see PCT Publication WO 91/03569),

The DNA coding sequences for various neuratrophin proteins, which can be expressed using the present invention, are available. See, Ullrich et al. (**Nature** 303:821 (1983); E.P. Publication 121,,338, 30 published October 10, 1984) regarding hNGF coding

35 4368 regarding a hBDNF cDNA clone and, e.g., Leibrock

regarding a human NT-3 c[illegible] clone and Malsonpierre et al. (Science 247:1446 (1990)) and Hohn et al. (**Nature** 344:339 (1990)) regarding NT-3 coding sequences from various other species. The cloning of the human (Rosenthal et al,, Neuron 4:767 (1990)) as well as rat (Maisonpierre et al., infra) NT-3 genes has been reported, Furthermore, the nucleotide and amino acid sequences for BDNF are disclosed in PCT Publication WO 91/03568, published March 21, 1991 and copending U.S, application Serial No. 570,657 filed August 20, 1990; the nucleotide and amino acid sequences for NT-3 are disclosed in PCT Publication WO 91/03569 published March 21, 1991 and copending application Serial No.

NGF and BDNF are basic proteins of approximately 120 amino acids that share about 501 amino acid sequence identity, including absolute conservation of six cysteine residues that, in active NGF,, have been shown to form three disulfide bridges (Bradshaw, A,, 1978, Ann, Rev. Biochem, 47:191-216; Leibrock et al,, 1989, **Nature** 341:149-52). Comparison of the sequences of NGF from evolutionarily divergent species has revealed that the amino acids flanking these cysteine residues comprise the most highly conserved regions of the molecule (Meier et al,, 1986, EMBO Jw 5:1489-93; Selby et al,, 1987, J, Neurosci.

Res, 18:293-S)o StrikinglYr these -are also the regions which are most similar between BDNF and NGF (Leibrock et al,, 1989, **Nature** 341:149
In a preferred aspect of the present invention, a mature human neurotrophin is produced by expression of a chimeric prepro molecule according to the present invention. in a specific embodiment, the chimeric prepro molecule is encoded by a nucleic acid containing the long prepro region of NGF fused in frame to the coding sequence for mature BDNF, In another embodiment, the chimeric prepro molecule is encoded by a nucleic acid containing the prepro region of NT-3 fused in frame to the coding region for mature
- 19
BDNF. In yet another embodiment, the long prepro region of NGF is fused in frame to the coding region for NT-3,
As discussed supra, no distinct biological
5 significance between the long and short prepro region of the NGF precursor has been documented. In another specific aspect of the invention, either the long or short prepro region may be utilized in the construction of chimeric neurotrophic genes. one of
10 ordinary skill in the art can utilize either a short NGF prepro region or a long NGF prepro region when constructing chimeric fusions of the present invention comprising an NGF prepro region.

Due to the degeneracy of nucleotide coding sequences, other DNA sequences that encode substantially the same

[illegible] functionally equivalent amino acid residue within the sequence, thus producing a silent change. Likewise, [illegible]

to, those containing, as part of their primary amino acid sequence, altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a 5functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobia) amino acids include 10 alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threoniner cysteiner tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids 15 include **arginine**, lysine and histidine, The negatively charged (acidic) amino acids include aspartic acid and glutamic acid, Also included within the scope of the invention are neurotrophin proteins or fragments or derivatives thereof which are obtained 20 through modification during or after translation, e.q,, by glycosylation, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, acetylation, phosphorylation, reduction, cleavage, etc.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a sequence encoding a chimeric neurotrophic prepro protein or prepro peptide, consisting of appropriate 10 transcriptional/translational control signals upstream of the chimeric DNA sequences, These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (genetic recombination), Expression of nucleic acid sequences 15 encoding chimeric neurotrophic prepro protein or prepro peptide may be regulated by a second nucleic acid sequence so that chimeric neurotrophic prepro protein or prepro peptide is expressed in a host transformed with the recombinant DNA molecule, For 2G example, expression may be controlled by any promoter/enhancer element known in the art to be active in mammalian cells, Promoters which may be used to control chimeric neurotrophic factor expression include, but are not limited to, the cytomegalovirus (CMV) promoter,, the SV40 early promoter region (Bernoist and Chambon, 1981, **Nature** 290:304-310), the promoter contained in the 31 long terminal repeat of Rous sarcoma virus (Yamamoto, et al,, 1980, Call 22:787-797), the herpes thymidine kinase promoter (Wagner et al,, 1981, Proc, Natl, Acad. Sci, U,S.A, 78:144-1445). the regulatory sequences of the metallothionine gene (Brinster et al,, 1982, **Nature** 296:39-42); and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic

Hepat[illegible] which is active in pancreatic beta cells Hanahan, 1985, **Nature** 315:115 122 , immunoglobulin gene cont[illegible]

10 **Nature** 318:533-538; Alexander et al., 1987, Mol. Cell

5:1639-1648; Hammer et al., 1987, Science 235:53-58); alpha 1-antitrypsin gene control region which is 2o active in the liver (Kelsey et al, 1987, Genes and Devel, 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, **Nature** 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al,, 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, **Nature** 314:283-286), and gonadotropic releasing hormone gene control region which is active -30 in the hypothalamus (Mason et al,, 1986, Science 234:1372-1378),

A specific example of an expression vector which can be used is CDM8 (Seed, 1987, **Nature** 329:840-842; Seed and Aruffo, 1987, Proc. Natl, Acad, Sci. USA 84:3365-3369; Aruffo & Seed, Proc. Natl, Acad, Sci.

10 The preparation of the vector was carried out by digesting pC81mN (long mouse NGF in pCDM8) with both Eco47 and Notl and isolating the 4,6 kb vector fragment by gel electrophoresis. The 365 bp fragment was ligated into the Eco47/Notl sites of pC81m.N. This 15 ligation resulted in a direct in frame fusion of the mouse NGF prepro region with the mature BDNF coding region, Constructs were diagnostically tested by digesting with BssH2. by assessing the loss of the Eco47 site during the subcloning, and ultimately by 2o DNA sequencing,

6,2. EXPRESSION OF CHIMERIC MOLECULES

CHO-DG44 cells were used to generate stable lines for the production of bioactive BDNF. CHO-DG44 cells (obtained from Dr. L, Chasin at Columbia University) lack both copies of the dihydrofolate reductase gene (Urlaub and Chasin, 1980, Proc, Natl, Acad. Sci, USA 77:4216-4220). Stably transfected CHO-OG44 cell lines expressing BDNF have been previously described (PCT International Publication No. WO 91/03568, published March 21, 1991). These lines were generated by transfection with pC8hB DNA which encodes the human BDNF gene including the prepro region cloned into the expression vector pCDM8. CHO-DG44 cells (1 X 106 cells/100 mm plate) were transfected by the calcium phosphate coprecipitation method with 20 gg of the NGF/BDNF chimera (pC81mN/B) along with 0,2 gq of plasmid p410 which encodes a weakened dihydrofolate reductase gene (dhfr), 48 5hours after transfection, the cells were passaged into selection media (Ham's F12 without hypoxanthine and thymidine containing 10% dialyzed fetal bovine serum and 1% each of penicillin and streptomycin; -HT media). -HT-resistant clones were treated as pools 10 for amplification with methotrexate (MTX). Clones obtained with 0,05 gM MTX were also treated as pools for further amplification at 2,5 gM MTX. A single

assessed by scoring neurite outgrowth of embryonic [illegible] chick dorsal root ganglia (DRG) Maisonpierre et

EFFICIENCY BETWEEN HOMOLOGUS PREPRO
BDNF AND PREPRO NGF/BDNF CHIMERA
Experiments were performed to directly
25 compare the processing and expression of preproBDNF
with the **preproNGF**/BDNF chimera in CHO cells.

15 Unprocessed proBDNF (31 kD), the pro-portion of the processed proBDNF precursor (16 kD) and the mature form (14 kD) of the short preproBDNF protein were detected in the stably transfected cell line DGZ1000-B 2.5 (obtained after similar MTX selection and
20 amplification as used for cell line DGC-N/B 5-#23) (Figure 1. lane 3), Only the proteolytically processed mature form of BDNF (14 kD) was detected in DGC-N/B 5-#23, stably transfected with the long **proNGF**/BDNF chimeric construction (Figure 1. lane 4).

25- Unprocessed **proNGF**/BDNF was not detected in the conditioned media from this cell line, We estimate from the intensity of the labeling of the mature BDNF that cell line DGC-N/B 5-#23 produced about five (5) times as much mature BDNF protein per cell relative to 30 cell line DGZ1000 B 2.5 made with the short proBDNF construct.

TABLE 1
Effect of Various COS
SuRernatants on DRG Egplants
SAMPLE DILUTION DRG
CONTROL 0f0j0j0f0.5
NGF 10 ng/ml 5+r5+15+f5+f5+
MOCK 10 ul ofilififl
50 Al 0.5rlflflfl.5
100 jul 0.510*5flflfl
250 gl 2f2,5F2.5f2,5f2,5
smNGF 10 Jul 2r3f3p3f3,5
50 Jul 5r5f5f5f5
100 41 5+f5+f5+f5+f5+
250 ul 5+f5+15+f5+15+
lmNGF 10 Al 2,2f2l2f2
5 0 1 4 f 4 F 4 r 4 f 4
100)Ul 5r5f5f515
1250 gl 5f5f5F515
,4. CONCLUSIONS
We conclude from these studies that the long pro portion of NGF is better suited for the processing
5 of BDNF in CHO cells than the short pro portion of BDNF. The advantages of the chimeric **proNGF**/mature BDNF gene construct, therefore, is that it allows for higher expression levels of BDNF on a per cell basis in mammalian cells. Additionally, it should allow for
10 better purification schemes for BDNF in that contaminating unprocessed forms of BDNF are not apparent in the crude supernatants,
Additionally, use of either the long or short prepro region of NGF results in the expression
15 of biologically active NGF. This indicates that

[illegible] BDNF CHIMERA
[illegible] CONSTRUCTION OF CHIMERIC NUCLEIC ACID MOLECULES
A HindIII XhoI DNA fragment containing the
[illegible]

=> d l8 1-19

L8 ANSWER 1 OF 19 USPATFULL
AN 2002:251935 USPATFULL
TI Purification of NGF
IN Burton, Louis E., San Mateo, CA, UNITED STATES
Schmelzer, Charles H., Burlingame, CA, UNITED STATES
Beck, Joanne T., Westlake Village, CA, UNITED STATES
PI US 2002137893 A1 20020926
AI US 2002-72681 A1 20020208 (10)
RLI Continuation of Ser. No. US 2000-675503, filed on 29 Sep 2000, GRANTED, Pat. No. US 6423831 Continuation of Ser. No. US 1999-363573, filed on 29 Jul 1999, GRANTED, Pat. No. US 6184360 Continuation of Ser. No. US 1997-970865, filed on 14 Nov 1997, GRANTED, Pat. No. US 6005081
PRAI US 1996-30838P 19961115 (60)
US 1997-47855P 19970529 (60)
DT Utility
FS APPLICATION
LN.CNT 2052
INCL INCLM: 530/350.000
INCLS: 530/417.000
NCL NCLM: 530/350.000
NCLS: 530/417.000
IC [7]
ICM: C07K014-435
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L8 ANSWER 2 OF 19 USPATFULL
AN 2002:85534 USPATFULL
TI NOVEL NEUROTROPHIC FACTOR
IN ROSENTHAL, ARNON, PACIFICA, CA, UNITED STATES
PI US 2002045576 A1 20020418
US 6506728 B2 20030114
AI US 1995-450842 A1 19950526 (8)
RLI Division of Ser. No. US 1995-426419, filed on 19 Apr 1995, ABANDONED Continuation of Ser. No. US 1993-30013, filed on 22 Mar 1993, ABANDONED A 371 of International Ser. No. WO 1991-US6950, filed on 24 Sep 1991, UNKNOWN Continuation-in-part of Ser. No. US 1991-648482, filed on 31 Jan 1991, ABANDONED Continuation-in-part of Ser. No. US 1990-587707, filed on 25 Sep 1990, GRANTED, Pat. No. US 5364769
DT Utility
FS APPLICATION
LN.CNT 2815
INCL INCLM: 514/012.000
INCLS: 514/002.000
NCL NCLM: 514/012.000
NCLS: 514/002.000
IC [7]
ICM: A01N037-16
ICS: A61K038-17
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L8 ANSWER 3 OF 19 USPATFULL
AN 2002:181791 USPATFULL
TI Isolation of neurotrophins from a mixture containing other proteins and neurotrophin variants using hydrophobic interaction chromatography
IN Burton, Louis E., San Mateo, CA, United States
Schmelzer, Charles H., Burlingame, CA, United States
[illegible]
filed on 14 Nov [illegible], now patented, Pat. No. [illegible]
PRAI US 1997-47855P 19970529 (60)
[illegible]

.FS GRANTED
LN.CNT 2348
INCL INCLM: 530/399.000
INCLS: 530/324.000; 530/350.000; 530/412.000; 530/416.000; 530/417.000; 435/069.100; 435/069.400; 435/070.100; 435/071.100
NCL NCLM: 530/399.000
NCLS: 435/069.100; 435/069.400; 435/070.100; 435/071.100; 530/324.000; 530/350.000; 530/412.000; 530/416.000; 530/417.000
IC [7]
ICM: C07K003-14
ICS: C12P021-06
EXF 530/399; 530/324; 530/350; 530/412; 530/416; 530/417; 435/69.1; 435/69.4; 435/70.1; 435/71.1
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L8 ANSWER 4 OF 19 PCTFULL COPYRIGHT 2003 Univentio
AN 2002044203 PCTFULL ED 20020624 EW 200223
TIEN PRODUCTION OF RECOMBINANT BMP-2
TIFR PRODUCTION DE BMP-2 RECOMBINEE
TIDE HERSTELLUNG VON REKOMBINANTEM BMP-2
IN RUDOLPH, Rainer, Dr.-Hans-Litten-Str. 28, 06120 Halle, DE [DE, DE];
SCHWARZ, Elisabeth, Dr.-Hans-Litten-Str. 28, 06120 Halle, DE [DE, DE];
HERR, Gerhard, Turmstrasse 16, 35578 Wetzlar, DE [DE, DE];
HILLGER, Frank, Fischereiweg 32, 06846 Dessau, DE [DE, DE]
PA SCIL PROTEINS GMBH, Heinrich-Damerow-Strasse 1, 06120 Halle, DE [DE, DE], for all designates States except US;
RUDOLPH, Rainer, Dr.-Hans-Litten-Str. 28, 06120 Halle, DE [DE, DE], for US only;
SCHWARZ, Elisabeth, Dr.-Hans-Litten-Str. 28, 06120 Halle, DE [DE, DE], for US only;
HERR, Gerhard, Turmstrasse 16, 35578 Wetzlar, DE [DE, DE], for US only;
HILLGER, Frank, Fischereiweg 32, 06846 Dessau, DE [DE, DE], for US only
AG WEICKMANN & WEICKMANN, Postfach 860 820, 81635 Muenchen, DE
LAF German
LA German
DT Patent
PI WO 2002044203 A2 20020606
DS W: AE AG AL AM AT AU AZ BA BB BG BR BY BZ CA CH CN CO CR CU CZ DE DK DM DZ EC EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX MZ NO NZ OM PH PL PT RO RU SD SE SG SI SK SL TJ TM TR TT TZ UA UG US UZ VN YU ZA ZM ZW
RW (ARIPO): GH GM KE LS MW MZ SD SL SZ TZ UG ZM ZW
RW (EAPO): AM AZ BY KG KZ MD RU TJ TM
RW (EPO): AT BE CH CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE TR
RW (OAPI): BF BJ CF CG CI CM GA GN GQ GW ML MR NE SN TD TG
AI WO 2001-EP13840 A 20011127
PRAI DE 2000-100 59 336.4 20001129
ICM C07K014-00

L8 ANSWER 5 OF 19 USPATFULL
AN 2001:18606 USPATFULL
TI Purification of NGF
IN Burton, Louis E., San Mateo, CA, United States
Schmelzer, Charles H., Burlingame, CA, United States
Beck, Joanne T., Westlake Village, CA, United States
PA Genentech, Inc., South San Francisco, CA, United States (U.S. corporation)
PI US 6184360 B1 20010206
[illegible]

[illegible]
FS Granted
LN.CNT 2226
[illegible]

435/069.100; 435/069.400; 435/070.100; 435/071.100
NCL NCLM: 530/399.000
NCLS: 435/069.100; 435/069.400; 435/070.100; 435/071.100; 530/324.000; 530/350.000; 530/412.000; 530/416.000; 530/417.000
IC [7]
ICM: C07K003-14
ICS: C12P021-06
EXF 530/399; 530/324; 530/350; 530/412; 530/416; 530/417; 435/69.1; 435/69.4; 435/70.1; 435/71.1
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L8 ANSWER 6 OF 19 USPATFULL
AN 2001:7868 USPATFULL
TI Neuronal factor
IN Rosenthal, Arnon, Pacifica, CA, United States
Winslow, John W., El Granada, CA, United States
PA Genentech, Inc., So. San Francisco, CA, United States (U.S. corporation)
PI US 6174701 B1 20010116
AI US 1995-455741 19950531 (8)
RLI Continuation of Ser. No. US 1995-381030, filed on 31 Jan 1995 Continuation of Ser. No. US 1990-494024, filed on 15 Mar 1990, now abandoned Continuation-in-part of Ser. No. US 1989-449811, filed on 12 Dec 1989, now abandoned
DT Utility
FS Granted
LN.CNT 1480
INCL INCLM: 435/069.100
INCLS: 536/023.500; 435/320.100; 435/325.000; 435/352.000; 435/354.000; 435/357.000; 435/358.000; 435/364.000; 435/366.000; 435/367.000; 435/252.300; 435/252.330; 435/069.700; 435/069.800
NCL NCLM: 435/069.100
NCLS: 435/069.700; 435/069.800; 435/252.300; 435/252.330; 435/320.100; 435/325.000; 435/352.000; 435/354.000; 435/357.000; 435/358.000; 435/364.000; 435/366.000; 435/367.000; 536/023.500
IC [7]
ICM: C12N015-00
ICS: C12N005-02; C12P021-06; C07H021-04
EXF 435/6; 435/69.1; 435/69.8; 435/240.2; 435/252.3; 435/252.33; 435/320.1; 435/325; 435/352; 435/354; 435/357; 435/358; 435/364; 435/366; 435/367; 536/23.5; 536/24.3; 536/24.31; 536/24.33
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L8 ANSWER 7 OF 19 USPATFULL
AN 2000:31394 USPATFULL
TI Neurotrophic factor (NT-4)
IN Rosenthal, Arnon, Pacifica, CA, United States
PA Genentech, Inc., South San Francisco, CA, United States (U.S. corporation)
PI US 6037320 20000314
AI US 1997-928694 19970912 (8)
RLI Continuation of Ser. No. US 1995-451947, filed on 26 May 1995, now patented, Pat. No. US 5702906 which is a division of Ser. No. US 1995-426419, filed on 19 Apr 1995, now abandoned which is a continuation of Ser. No. US 30013
DT Utility
FS Granted
LN.CNT 2746
INCL INCLM: 514/002.000
INCLS: 514/012.000; 530/350.000
NCL NCLM: 514/002.000

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

TIEN MAMMALIAN SUBTILISIN/KEX[redacted] ISOZYME SKI-1: A PROPROTEIN C[redacted]ERTASE WITH A UNIQUE CLEAVAGE SPECIFIC[redacted]Y
TIFR SUBTILISINE/KEXINE ISOZYME SKI-1 MAMMIFERE : PROPROTEINE CONVERTASE DOTEE D'UNE SPECIFICITE DE CLIVAGE UNIQUE
IN SEIDAH, Nabil;
CHRETIEN, Michel;
MARCINKIEWICZ, Mieczyslaw;
LAAKSONEN, Reijo;
DAVIGNON, Jean
PA INSTITUT DE RECHERCHES CLINIQUES DE MONTREAL;
SEIDAH, Nabil;
CHRETIEN, Michel;
MARCINKIEWICZ, Mieczyslaw;
LAAKSONEN, Reijo;
DAVIGNON, Jean
LA English
DT Patent
PI WO 2000026348 A2 20000511
DS W: AE AL AM AT AU AZ BA BB BG BR BY CA CH CN CR CU CZ DE DK DM
EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ
LC LK LR LS LT LU LV MA MD MG MK MN MW MX NO NZ PL PT RO RU
SD SE SG SI SK SL TJ TM TR TT TZ UA UG US UZ VN YU ZA ZW GH
GM KE LS MW SD SL SZ TZ UG ZW AM AZ BY KG KZ MD RU TJ TM AT
BE CH CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE BF BJ CF
CG CI CM GA GN GW ML MR NE SN TD TG
AI WO 1999-CA1058 A 19991104
PRAI CA 1998-2,249,648 19981104
ICM C12N009-64
ICS C07K014-81

L8 ANSWER 9 OF 19 PCTFULL COPYRIGHT 2003 Univentio
AN 2000022119 PCTFULL ED 20020515
TIEN METHOD FOR OBTAINING ACTIVE β-NGF
TIFR PROCEDE D'OBTENTION DE NGF-β ACTIF
IN RUDOLPH, Rainer;
RATTENHOLL, Anke;
SCHWARZ, Elisabeth;
GROSSMANN, Adelbert
PA RUDOLPH, Rainer;
RATTENHOLL, Anke;
SCHWARZ, Elisabeth;
GROSSMANN, Adelbert
LA German
DT Patent
PI WO 2000022119 A1 20000420
DS W: AU BR CA JP KR US ZA
AI WO 1999-EP7613 A 19991011
PRAI EP 1998-98119077.0 19981009
ICM C12N015-12
ICS C07K014-48

L8 ANSWER 10 OF 19 USPATFULL
AN 1999:167121 USPATFULL
TI Purification of recombinant human neurotrophins
IN Burton, Louis E., San Mateo, CA, United States
Schmelzer, Charles H., Burlingame, CA, United States
Beck, Joanne T., Westlake Village, CA, United States
PA Genentech, Inc., South San Francisco, CA, United States (U.S. corporation)
PI [redacted]

[redacted]
LN.CNT 2397
INCL INCLM: 530.399.000

NCL NCLM: 530/399.000
NCLS: 435/069.100; 435/[illegible]9.400; 435/070.100; 435/071.100; 530/324.000; 530/350.000; 530/412.000; 530/416.000; 530/417.000
IC [6]
ICM: C07K003-14
ICS: C12P021-06
EXF 530/399; 530/324; 530/350; 530/412; 530/416; 530/417; 435/69.1; 435/69.4; 435/70.1; 435/71.1
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L8 ANSWER 11 OF 19 USPATFULL
AN 1999:92541 USPATFULL
TI Protein expression system
IN Sgarlato, Gregory D., Los Gatos, CA, United States
PA Technologene, Inc., Los Gatos, CA, United States (U.S. corporation)
PI US 5935824 19990810
AI US 1996-595043 19960131 (8)
DT Utility
FS Granted
LN.CNT 5959
INCL INCLM: 435/069.700
INCLS: 435/069.800; 530/350.000; 536/023.400
NCL NCLM: 435/069.700
NCLS: 435/069.800; 530/350.000; 536/023.400
IC [6]
ICM: C07K019-00
ICS: C12N015-62
EXF 435/69.7; 435/69.8; 435/207; 435/68.1; 436/532; 436/828; 530/387.1; 530/350; 530/413; 530/812; 530/866; 530/867; 536/23.4; 536/23.2; 536/23.53; 536/23.7; 935/47
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L8 ANSWER 12 OF 19 USPATFULL
AN 1998:135007 USPATFULL
TI Neurotrophic factor
IN Rosenthal, Arnon, Pacifica, CA, United States
PA Genentech, Inc., South San Francisco, CA, United States (U.S. corporation)
PI US 5830858 19981103
AI US 1995-424826 19950419 (8)
RLI Continuation of Ser. No. US 1994-240387, filed on 10 May 1994, now abandoned which is a continuation of Ser. No. US 1991-648482, filed on 13 Jan 1991, now abandoned which is a continuation-in-part of Ser. No. US 1990-587707, filed on 25 Sep 1990, now patented, Pat. No. US 5364769
DT Utility
FS Granted
LN.CNT 2363
INCL INCLM: 514/012.000
INCLS: 514/002.000; 530/350.000; 530/395.000; 530/399.000; 530/402.000; 435/069.100
NCL NCLM: 514/012.000
NCLS: 435/069.100; 514/002.000; 530/350.000; 530/395.000; 530/399.000; 530/402.000
IC [6]
ICM: A61K038-18
ICS: C07K014-475
EXF 514/2; 514/12; 530/350; 530/395; 530/399; 530/402; 435/69.1
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L8 ANSWER 13 OF 19 USPATFULL

PI US 5702906 19971230
AI US 1995-451947 19950526 (8)

abandoned which is a continuation-in-part of Ser. No. US 1991-648482, filed on 31 Jan 1991, now abandoned which is a continuation-in-part of Ser. No. US 1990-587707, filed on 25 Sep 1990, now patented, Pat. No. US 5364769
DT Utility
FS Granted
LN.CNT 2046
INCL INCLM: 435/007.100
INCLS: 530/387.100; 530/387.900; 530/388.240; 530/413.000; 435/336.000; 435/236.000
NCL NCLM: 435/007.100
NCLS: 435/236.000; 435/336.000; 530/387.100; 530/387.900; 530/388.240; 530/413.000
IC [6]
ICM: G01N033-53
ICS: C12N005-12; C07K016-22; C07K001-16
EXF 424/139.1; 424/141.1; 424/145.1; 424/9.1; 435/7.1; 435/336; 530/387.1; 530/387.9; 530/388.1; 530/388.15; 530/388.24; 530/389.1; 530/389.2; 530/391.3; 530/413
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L8 ANSWER 14 OF 19 PCTFULL COPYRIGHT 2003 Univentio
AN 1997028272 PCTFULL ED 20020514
TIEN PROTEIN EXPRESSION SYSTEM
TIFR SYSTEME D'EXPRESSION DE PROTEINES
IN SGARLATO, Gregory, D.
PA TECHNOLOGENE INC.
LA English
DT Patent
PI WO 9728272 A1 19970807
DS W: AU CA JP AT BE CH DE DK ES FI FR GB GR IE IT LU MC NL PT SE
AI WO 1997-US1470 A 19970131
PRAI US 1996-8/595,043 19960131
ICM C12P021-00
ICS C12P021-06; C07K001-12; C07K001-22; C07K014-46; C07K014-195; C07K016-00; C07K019-00; C07H021-04; C12N009-38

L8 ANSWER 15 OF 19 USPATFULL
AN 94:99824 USPATFULL
TI Nucleic acid encoding neurotrophic factor four (NT-4), vectors, host cells and methods of production
IN Rosenthal, Arnon, Pacifica, CA, United States
PA Genentech, Inc., South San Francisco, CA, United States (U.S. corporation)
PI US 5364769 19941115
AI US 1990-587707 19900925 (7)
DT Utility
FS Granted
LN.CNT 1357
INCL INCLM: 435/069.100
INCLS: 435/069.400; 435/320.100; 435/240.100; 435/240.200; 536/023.500; 536/023.510
NCL NCLM: 435/069.100
NCLS: 435/069.400; 435/320.100; 435/369.000; 536/023.500; 536/023.510
IC [5]
ICM: C12N005-10
ICS: C12N015-18; C12N015-12
EXF 536/27; 536/23.50; 536/23.51; 536/252.3; 435/69.1; 435/69.4; 435/320.1; 435/240.1; 435/240.2
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L8 [illegible]
TI NEUROTROPHINE 4
IN IP, Nancy;

VENTIMIGLIA, Roseann;
WIEGAND, Stanley;
WONG, Vivien;
YANCOPOULOS, George, D.
PA REGENERON PHARMACEUTICALS, INC.;
IP, Nancy;
ALTAR, Charles, A.;
DISTEFANO, Peter;
VENTIMIGLIA, Roseann;
WIEGAND, Stanley;
WONG, Vivien;
YANCOPOULOS, George, D.
LA English
DT Patent
PI WO 9325684 A1 19931223
DS W: AU BB BG BR BY CA CZ FI HU JP KR KZ LK MG MN MW NO NZ PL RO
RU SD SK UA US AT BE CH DE DK ES FR GB GR IE IT LU MC NL PT
SE BF BJ CF CG CI CM GA GN ML MR NE SN TD TG
AI WO 1993-US5672 A 19930611
PRAI US 1992-898,194 19920612
ICM C12N015-12
ICS C12Q001-68; C12P021-08; A61K037-02

L8 ANSWER 17 OF 19 PCTFULL COPYRIGHT 2003 Univentio
AN 1992020365 PCTFULL ED 20020513
TIEN THERAPEUTIC AND DIAGNOSTIC METHODS BASED ON NEUROTROPHIN-4 EXPRESSION
TIFR PROCEDES THERAPEUTIQUES ET DIAGNOSTIQUES BASEES SUR L'EXPRESSION DE NEUROTROPHINE-4
IN HALLBOOK, Finn;
IBANEZ MOLINER, Carlos, Fernando;
PERSSON, Hakan, Bengt;
IP, Nancy;
YANCOPOULOS, George, D.
PA REGENERON PHARMACEUTICALS, INC.;
HALLBOOK, Finn;
IBANEZ MOLINER, Carlos, Fernando;
PERSSON, Hakan, Bengt
LA English
DT Patent
PI WO 9220365 A1 19921126
DS W: AT AU BE CA CH CS DE DK ES FI FR GB GR HU IT JP KR LU MC NL
NO RU SE
AI WO 1992-US4266 A 19920520
PRAI US 1991-703,450 19910521
US 1991-729,253 19910712
US 1991-734,422 19910723
US 1991-751,356 19910828
US 1991-762,674 19910920
US 1991-791,924 19911114
ICM A61K037-02
ICS A61K049-00; G01N033-50; G01N033-68; C07K039-00; C12N015-12; C12N015-79

L8 ANSWER 18 OF 19 PCTFULL COPYRIGHT 2003 Univentio
AN 1992005254 PCTFULL ED 20020513
TIEN NOVEL NEUROTHROPHIC FACTOR
TIFR NOUVEAU FACTEUR NEUROTROPHIQUE
IN ROSENTHAL, Arnon
PA GENENTECH, INC.;
ROSENTHAL, Arnon

AI US 1990 581,707 19900925
PRAI US 1991 648,482 19910131

L8 ANSWER 19 OF 19 MEDLINE DUPLICATE 1
AN 89178770 MEDLINE
DN 89178770 PubMed ID: 2648014
TI Synthesis of chimeric mouse nerve growth factor precursor and human beta-nerve growth factor in Escherichia coli: immunological properties.
AU Dicou E; Houlgatte R; Lee J; von Wilcken-Bergmann B
CS INSERM U, Centre Hospitalier Regional, Angers, France.
SO JOURNAL OF NEUROSCIENCE RESEARCH, (1989 Jan) 22 (1) 13-9.
Journal code: 7600111. ISSN: 0360-4012.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 198904
ED Entered STN: 19900306
Last Updated on STN: 19900306
Entered Medline: 19890427

=> d 9,19 hit
15 ANSWERS ARE AVAILABLE. SPECIFIED ANSWER NUMBER EXCEEDS ANSWER SET SIZE
The answer numbers requested are not in the answer set.
ENTER ANSWER NUMBER OR RANGE (1):end

=> d l8 9,19 hit

L8 ANSWER 9 OF 19 PCTFULL COPYRIGHT 2003 Univentio
ABEN The invention relates to a method for producing biologically active β-NGF from the proform
proNGF. After expressing the proform of the β-NGF in a prokaryotic host cell, the recombinant protein is isolated in the form of insoluble inactive aggregates (inclusion bodies). After the solubilization thereof in a strong **denaturing** agent and the subsequent conversion thereof into the natural conformation, which is determined by the disulfide bridges present in the natural β-NGF, biologically active β-NGF is obtained by subsequently splitting-off the prosequence.
ABFR L'invention concerne un procede de preparation de NGF-β biologiquement actif, a partir de la proforme **proNGF**. Apres expression de la proforme du NGF-β dans une cellule hote procaryote, la proteine de recombinee est isolee sous forme d'ensembles inactifs insolubles (corps d'inclusion). Apres leur solubilisation dans un agent de **denaturation** puissant puis leur conversion a la conformation naturelle qui est determinee par les ponts disulfure presents dans le NGF-β naturel, le NGF-β biologiquement actif est obtenu par separation de la prosequence.

DETD Verfahren zur Gewinnung von aktivem ss-NGF
Die vorliegende Erfindung betrifft ein Verfahren zur Gewinnung von ss-NGF durch Naturie-
rung von denaturiertem, inaktiven **proNGF** und Abspaltung der Prosequenz.

Proform **proNGF**
Inclusion Bodies nach

dass der **proNGF** in seiner inaktiven schwer loeslichen Form mit einer Loesung eines Denaturierungsmittels in einer denaturierenden Konzentration geloest wird, anschliessend unter Erhalt der Loeslichkeit in eine nicht oder schwach denaturierende Loesung ueberfaehrt wird und dabei geloester denaturierter **proNGF** eine biologisch aktive Konformation annimmt, die durch die im natuerlichen NGF vorliegenden Disulfidbruecken bestimmt ist und anschliessend die Prosequenz abgespalten wird, wobei aktiver NGF erhalten wird, der isoliert werden kann.

Unter **proNGF** ist ss-NGF zu verstehen, welcher am N-Terminus mit seiner Prosequenz verknuepft ist. Erfindungsgemaess kann als Prosequenz entweder die gesamte Prosequenz (US-Patent 5,683,894; Ulrich, A. et al. Nature 303 (1983) 821; SWISS-PROT Protein Sequence Database No. P01138) oder Teile davon, vorzugsweise vollstaendige Domaenen, verwendet werden. Suter et al. (ENMO J. 10 2395 (1991» haben die in vivo-Funktion des Propeptids von murinem ss-NGF hinsichtlich der korrekten Sekretion in einem COS Zellkultursystem naeher untersucht. Dazu wurde die Prosequenz in fuenf Bereiche eingeteilt. Es wurden Mutanten hergestellt, in denen ein oder mehrere dieser Sequenzen deletiert wurden. Dabei wurde gefunden, dass die Sequenzbereiche mit den Aminosaeuren -52 bis -26 (Domaene F') sowie -6 bis -1 (Domaene II) fuer die Expression und Sekretion von biologisch aktivem ss-NGF essentiell sind. Domaene 1 ist wichtig fuer die Expression, waehrend Domaene II die korrekte proteolytische Prozessierung bewirkt. Es hat sich ueberraschenderweise gezeigt, dass **proNGF** in analoger Weise wie ss-NGF eine Aktivitaet in vivo zeigt. **proNGF** kann damit ebenfalls als Therapeutikum verwendet werden.

Inaktiver, schwer loeslicher **proNGF** entsteht bei der Ueberexpression des Proteins im Cytosol von Prokaryoten. Rekombinant hergestellter **proNGF** verbleibt dabei in unloeslicher und aggregierter Form im Cytoplasma. Derartige Aggregate von Proteinen, deren Isolierung und Reinigung sind beispielsweise in Marston, F.A., Biochem. J. 240 (1986) 1 beschrieben. Zur Isolierung dieser inaktiven Proteinaggregate (Inclusion Bodies) werden die prokaryotischen Zellen nach der Fermentation aufgeschlossen.

Die Prosequenz stellt eine vom reifen Protein getrennte Domaene dar. Zwischen diesen Domaenen befindet sich eine exponierte Proteasespaltstelle. Diese Spaltstellen lassen sich spe

proNGF

wird durch [illegible] abgebaut. **Denaturierte** Proteine und auch Faltungsintermediate mit gegen exponieren Sequenzen,

proNGF

Proteasen mit Trypsin-aehnlicher Substratspezifitaet bevorzugt. Diese Proteasen spalten das Protein, ohne den aktiven Teil des Proteinmolekuels abzubauen. Als Trypsin-aehnliche Proteasen kommen verschiedene Serin-Poteasen (z.B. Trypsin selbst oder y-NGF) in Frage. Bevorzugt wird Trypsin eingesetzt. Fuer die limitierte Proteolyse wird das Protein in einem Masse-Verhaeltnis von 1:40 bis 1:2500 (Verhaeltnis Trypsin : **proNGF**) eingesetzt, bevorzugt wird ein Bereich von 1:40 bis 1:250. Die Proteolyse wird mit einer Inkubationszeit von 1 min - 24 h, bevorzugt 1 - 60 min bei einer Temperatur von O'C bis 37'C, bevorzugt O'C bis 20'C, durchgefuehrt. Als Puffer werden solche verwendet, die die Aktivitaet der Protease nicht hemmen. Bevorzugt sind Phosphat- und Tris-Puffer im Konzentrationsbereich von 10-100 mM. Die linuetierte Proteolyse wird im Bereich des pH-Optimums der Protease durchgefuehrt, bevorzugt ist ein Milieu von pH 7
Nach Ende der Inkubationszeit wird die Proteolyse gestoppt, endweder durch Zugabe eines spezifischen Inhibitors, bevorzugt 1-5 mM PMSF (Phenylmethylsulfonylfluorid) oder Sojabohnen-Trypsininhibitor, bevorzugt 1 mg auf 0 5 mg Trypsin oder durch Erniedrigung des pH-Werts auf pH 2-3 durch Zugabe von Saeure, bevorzugt HCI (Rudolph, R. et al. (1997).

Figur 1 zeigt das **proNGF**-Plasmidkonstrukt pETlla-**proNGF** ffir die Expression von rekombinantem humanen **proNGF**.

Figur 2 zeigt eine Coomassie-Faerbung eines SDS-PAGE-Gels (15 %) mit Roh-extrakten des E. coli-Stamrnes BL2l(DE3) pET11a-**proNGF**/pUBS520 vor bzw. nach Induktion sowie einer IB-Praeparation (SDS-PAGE nach Laemmli, U.K., Nature 227 (1970) 680). U: Rohextrakt vor Induktion, L Rohextrakt nach vierstuendiger Induktion, P: IB-Pellet, S: loeslicher Ueberstand).

Figur 2a zeigt den Einfluss des pH-Werts auf die Faltung von rh-**proNGF** bei IO'C in 1 00 mM Tris/I-ICI, 1 M L-Arginin, 5 mM GSH, 1 mM GS SG, 5 mM EDTA.

Figur 2d zeigt den Einfluss verschiedener GSSG-Konzentrationen auf die **Renaturie**-rung von rh-**proNGF**. Die GSH-Konzentration betrug 5 mM. Die restlichen Faltungsparameter waren identisch mit denen bei der GSH-Variation ver-wendeten. Dargestellt sind die Durchschnittswerte aus zwei Messreihen.

Figur 2e zeigt den Einfluss verschiedener GdmCl-Gehalte auf die

der NDS-Faltungsausbeute. Dargestellt sind die Durchschnittswerte aus zwei Messreihen.

Figur 2f zeigt den Effekt unterschiedlicher Proteinkonzentrationen auf die Faltungs-
ausbeute von rh-**proNGF**. Die GdmCI-Konzentration betrug in allen
Ansaetzen 200 mM. Alle anderen Faltungsparameter waren identisch mit denen bei der GdmCI-Variation verwendeten. Dargestellt ist eine Messreihe.

Figur 3 zeigt das Elutionsprofil der Reinigung von rh-**proNGF** mittels Kationen-
Austauschchromatographie an Poros 20 HS (Perseptive Biosystems, Saeulenvolumen 1.7 ml).

Figur 4 zeigt ein SDS-PAGE-Gel (15 %, Silberfaerbung nach Nesterenko, M.V. et
al., J. Biochem. Biophys. Methods 28 (1994) 239) der Reinigung von rh-**proNGF** an Poros 20 HS (1: Auftrag rh-**proNGF**-
Renaturat, 2: Durchlauf, 3.

Figur 5 zeigt das UV-Spektrum von rh-**proNGF**.

Figur 6 zeigt ein IEX-EPLC-Elutionsdiagramm von rh-**proNGF** (Saeulenmaterial.

Figur 7 zeigt ein RP-HPLC-Elutionsdiagramm von rh-**proNGF** bei 220 nrn (Saeule
Poros 10 Rl; 100 mm x 4.6 mm; Perseptive Biosystems).

Figur 8 zeigt ein SDS-Gel (15 % Coomassie-Faerbung) der limitierten Proteolyse von
rh-**proNGF** mit Trypsin (M: 10 kDa-Marker, 1: rh-**proNGF**
-Standard, 2: rh-
ss-NGF-Standard, 3: Masseverhaeltnis Trypsin rh-**proNGF** = 1 : 40, 4: 1
100, 5: 1 : 250, 6: 1 : 500, 7: 1 : 1000, 8: 1 2000, 9: 1 : 2500, 10: Kon-
trolle ohne Trypsin, mit STI).

SEQ ID NO: 1 und 2 zeigen Oligonukleotide zur Konstruktion von pETI la-**proNGF**.

SEQ ID NO: 3 zeigt die Nukleotidsequenz der cDNA von humanem **proNGF** sowie
die Aminosaeuresequenz des Translationsprodukts.

Beispiel 1
Klonierung der **proNGF**-cDNA in einen Escherichia coli-Expressionsvektor
Fuer die Klonierung des **proNGF**-Konstrukts wurde das T7-Expressionssystem von Novagen
[illegible]

[illegible]
[illegible] proNGFs wird durch

Die cDNA fuer humanen **proNGF** wurde durch PCR-Amplifikation aus dem Vektor pMGL-SIG-**proNGF** von Boehringer Mannheim erhalten (PL-Nr. 1905). Durch Mutageneseprimer wurde am 5'-Ende der fuer **proNGF** codierenden DNA-Sequenz eine Ndel- und am Y-Ende eine B-aniM-Schnittstelle eingefuehrt. Das PCR-Produkt wurde in die NdeI/BaniEH-Schnittstelle der multiplen Klonierungsstelle des Vektors pET 1 la (Novagen) inseriert (Fig. 1).

Beispiel 2

a) Expression von humanem **proNGF** in E. coli

Fuer die Anzucht des rekombinanten Bakterienstamms wurde eine Uebemachtkultur bereitet.

Die Kultur wurde bei 37'C und 200-250 rpm geschuettelt, bis eine OD600 von 0 0.8 erreicht war. Danach wurde die Expression von **proNGF** mit 3 mM IPTG 4 h lang bei gleicher Temperatur induziert. Anschliessend wurden die Zellen durch Zentrifugation geerntet und entweder sofort aufgeschlossen oder bei -70'C eingefroren.

4 g IB-Pellet erhalten. Die Praeparationen enthielten stets etwa 90-95% rh-**proNGF** (Fig. 2).

Beispiel 3

a) Solubilisierung der lBs

400 mg IB-Pellet wurden in 2 ml Solubilisierungspuffer (100 nim Tris/HCI pH 8.0; 6 M GdmCl; 100 mM DTT; 10 mM EDTA) suspendiert, 2 h bei 25'C inkubiert und 30 min bei 13.000 rpm im Kuehlraum abzentrifugiert. Anschliessend wurde der Ueberstand abgenommen und mit 1 M HCI auf pH 3-4 gebracht. Das Solubilisat wurde dreimal gegen 300 rnl 6 M GdmC1 pH 4.0; 10 mM EDTA dialysiert, und zwar zweimal je 2 h bei 25'C und einmal ueber Nacht im Kuehlraum (12'C; 16-18 h). Die Proteinkonzentration wurde dann nach der Bradford-Methode bestimmt (Bradford, M.M., Anal. Biochem. 72 (1976) 248). Die Konzentration an rh-**proNGF** betrug zwischen 40 und 50 mg/ml.

b) Optimierung der Renaturierung von rh-**proNGF**

Zur Darstellung von biologisch aktivem rh-pro-NGF aus den in Beispiel 3a) hergestellten Solubilisaten wurden diese in verschiedene Renaturierungspuffer verduennt. Zur Ermittlung der optimalen Faltungsbedingungen wurden folgende Parameter in der angegebenen Reihenfolge variiert.

proNGF

bestimmten Zeiten je 9[illegible] der Faltungsansätze mit [illegible] versetzt und dadurch

Saeule und das IPLC-Syste[redacted]Beckman Gold mit 125NM Solvent [redacted]dule, 168 Detektor,
Autosampler 507 und Auswertesoftware Gold V 8. 1 O verwendet. Die erhaltenen Elutions-
peaks wurden mit dem Programm Peakfit, Version 2.01, gefittet. Zur quantitativen
Bestimmung der Ausbeuten wurde eine Eichgerade mit gereinigtem, nativen rh-**proNGF**
erstellt. Da die rh-**proNGF**-IBs sehr sauber waren, wurde bei der quantitativen Auswertung
die zur Renaturierung eingesetzte Gesamtproteinmenge mit der von rh-pro-NGF gleichge-
setzt. Bei den dargestellten Messergebnissen handelt es sich um Durchschnittswerte aus je zwei
Messungen.

Temperatur [IC] Endausbeute ['/ol Plateau erreicht Geschwindigkeits-
nach ca. konstante k [s-']
4 25.8 3.3 h 2.596 xI O' s-,
10 29.0 1.6 h 4.865 x101 s-,
15 22.4 1.1 11 6.399 x10-4 s-'
20 12.0 1.Oh 1.065 xI O-, s-,
25 11.4 0.8 h 1.935 x10-3 S-1
Tabelle 2
Her ist der Einfluss verschiedener GSH/GSSG (GSH = reduziertes Glutathion, GSSG = oxi-
diertes Glutathion) auf die Faltung von rh-**proNGF** dargestellt. Als Renaturierungspuffer
wurde
100 mM Tris/HCI pH 9.5,
1 M L-Arginin,
5 mM EDTA
verwendet. Die Faltungsdauer betrug 3 h bei IO'C. In der Tabelle sind die einzelnen
Faltungsansaetze nach abnehmender Ausbeute geordnet. Angegeben sind die durchschnittlichen
Ausbeuten aus zwei Messreihen.

Nr. des Ansatzes GSH/GSSG-Verhaeltnis [mM] Ausbeute
1 5/0.5 37.7
2 5/1 35.0
3 5/5 34.0
4 5/15 33.1
5 1/1 29.4
6 5/10 27.6
7 5/20 26.0
8 2.5/1 22.1
9 10/1 21.2
10 115 18.9
11 20/1 10.9
12 0/1 9.85
13 1 0/0 0
14 1 5/0 0

c) Renaturierung von rh-**proNGF** im praeparativen Massstab

Rh-**proNGF** wurde durch Verduennung in Faltungspuffer (100 mM Tris/TICI pH 9.5; 1 M L-
Arginin; 5 mM GSH; 0.5 mM GSSG; 5 mM EDTA) **renaturiert**. Dabei wurde bei einer

d) Reinigung mittels Renaturierungschromatographie

Das **Renaturat** wurde gegen 10 mM Na-Phosphat pH 7.0; 1 mM EDTA, [illegible]

wurde auf eine Poros 20 [redacted] HS-Saeule (1.7 ml) aufgetragen und mit einem linearen Salzgradienten eluiert (IEX-Puffer B.

mM Na-Phosphat pH 7.0; 1 M NaCl; 1 mM EDTA). Das Protein eluiert bei 980 mM NaC1
(Fig. 3). Nicht nativer rh-**proNGF** laesst sich nur unter denaturierenden Bedingungen von der
Saeule entfernen.

Beispiel 4
Charakterisierung von rh-**proNGF**
a) Konzentrations- und Molekulargewichtsbestimmung mit UV-Spektralphotometrie
Zur Konzentrationsbestimmung von rh-**proNGF** in den gereinigten Proben wurde ein UV-
Spektrum von 240 bis 340 nm von gegen 50 mM Na-Phosphat pH 7 3 1 mm EDTA dialy-
sierten Proben gemessen (Fig. 5; das Spektrum wurde aufgenommen mit einem Beckman DU
640 Spectrophotometer). Die rh-**proNGF**-Konzentration der Probe wurde aus der Absorption
bei 280 nm bestimmt. Fuer die Berechnung wurde ein theoretischer niolarer
Extinktionskoeffizient von 25680 1/(molxcm) (berechnet nach Gill, S.C. et al., Anal.

Biochem. 182 (1989) 319) und das Molekulargewicht von 24869 Da pro Monomer (berechnet
mit dem ExPASy-Programm pI/Mw und korrigiert fuer drei Disulfidbruecken) zugrundegelegt. Die durch das Spektrum ermittelten Werte stimmten gut mit den durch die
Bradford-Methode bestimmten Konzentrationen ueberein. Die Molekulargewichtsbestimmung
erfolgte mittels Elektrospray-Massenspektrometrie. Die theoretische Masse von
rekombinantem **proNGF** betraegt 24869 Da. Experimentell wurden 24871 Da erhalten.

b) Reinheitsanalyse und Molekulargewichtsbestimmung mit SDS-Polyacryl-amidgelelektrophorese
Es wurden 15%ige Polyacrylamid-Gele verwendet. Die Proben enthielten jeweils 1% (v/v) 2-
Mercaptoethanol. Rekombinanter humaner **proNGF** zeigt im SDS-Gel ein etwas hoeheres
apparentes Molekulargewicht als erwartet: ca. 30 kDa (statt 24.8 kDa) (Fig. 2).

c) - Reinheitsanalyse mit IEX-HPLC
24 gg (50 gl einer Probe mit 0.48 mg/ml rh-**proNGF**) Protein wurden auf eine mit 50 MM Na-
Phosphat pH 7.0; 1 mM EDTA aequilibrierte Poros 20 HS-Saeule aufgetragen (125 x 4 mm)
und bei einer Flussrate von 5 ml/min mit einem linearen Gradienten von 0 bis 100 % B (B = 50

proNGF proNGF

Konzentration von 0.21 in ml) wurden auf eine mit 0.13% (v/v) TFA aequilibrierte Poros 10 RI-Saeule (100 mm x 4.6 mm, Perseptive Biosystems) aufgetragen. Das Protein wurde bei einer Flussrate von 0.8 ml/min mit einem nicht-linearen Gradienten innerhalb von 33 min eluiert (0-4 min: 6%B; 4-9 min: 6-30%B; 9-24 min: 30-69%B; 24-25 min: 69-100%B; 25-30 min: 100%B). Als Laufinittel B wurde 0.1% (v/v) TFA in 80% (v/v) Acetonitril verwendet. Zur Detektion wurde die Absorption bei 220 nm. herangezogen (Becknian Gold-FWLC-System mit Auswertesoftware Gold V 8.10).

Nativer rh-**proNGF** eluierte in einem einzigen Peak bei einer Retentionszeit von 14.28 min (Fig. 7).

H2N-Met-Glu-Pro-His-Ser-Glu-Ser-Asn-Val

1) Biologische Aktivitaet des rekombinanten humanen **proNGF**

Die physiologische Aktivitaet von rh-**proNGF** wurde mit dem DRG-Test (= dorsal root ganglion assay) ueberprueft (Levi-Montalcini, R. et al. Cancer Res. 14 (1954) 49; Varon, S. et al., Meth. in Neurochemistry 3 (1972) 203). Dabei wird die Stimulierbarkeit und das Ueberleben von sensorischen Neuronen aus dissoziierten Dorsalwurzelganglien von 7-8 Tage alten Huehnerembryonen anhand der Ausbildung von Neuriten untersucht. Die rh-**proNGF**-Probe wurde mit Kulturmedium auf Konzentrationen von 0.019 bis 20.00 ng/ml eingestellt. Es wurden 15000 Neuronen pro Testansatz eingesetzt. Nach 48-stuendiger Inkubation bei 37'C wurde die Zahl der ueberlebenden Zellen bestimmt. Als Referenz wurde eine Loesung von rh-ss-NGF mit bekannter Konzentration hinzugezogen. Bei der quantitativen Auswertung bezieht man sich auf den sogenannten EC5o-Wert, d.h. dejenigen NGF-Konzentration, bei der die Haelfte aller Neuronen ueberleben. Fuer rh-**proNGF** ergibt sich ein EC5(-Wert von 0.369 ng/nfl.

Beruecksichtigt man die unterschiedlichen Molekulargewichte von rh-ss-NGF und rh-**proNGF**, so ergibt sich fluer reifen rh ss-NGF eine etwa doppelt so grosse biologische Aktivitaet wie fuer rh-**proNGF**.

Beispiel 5

a) Gewinnung von biologisch aktivem, reifen rh-ss-NGF durch limitierte Proteolyse von rh-**proNGF**

Menschlicher **proNGF** besitzt als letzte Aminosaeure der Prosequenz ein Arginin. Daher kann

[illegible] **proNGF** [illegible]

dialysiert. Nach der Dialyse wurde mittels Aufnahme des UV-Spektrums eine

[illegible] proNGF

eingesetzt. Davon wurden [redacted]ch
der Proteolyse 3 gg (entsp[redacted]icht 6 gl) mittels SDS-PAGE ana[redacted]siert. Als Trypsin-Stamm-
loesungen wurden 0. 1 gg/ml bzw. 0. 0 1 gig/ml verwendet. Die Konzentration des Sojabohnen-
Trypsin-Inhibitors (STI) betrug 1 mg/n-fl. Beide Proteine lagen als Lyophilisate vor (Hersteller.

Fuer die linuetierte Proteolyse wurden unterschiedliche Trypsin/rh-**proNGF**-Masseverhaeltnisse
eingesetzt (s. Tabelle 3). Nach dreissignuertuetiger Inkubation auf Eis wurde die Reaktion mit je
gg STI abgestoppt. Als Kontrolle wurde rh-**proNGF** ohne Zugabe von Protease ebenfalls
auf Eis inkubiert und anschliessend mit STI versetzt.

Tabelle 3
Verhaeltnis M(Trypsin) V(Trypsin) [gl] V(rh-**proNGF**) [gl V(STI) [gl]
Trypsin:rh-**proNGF** ggl 1
1 :40 0.5 5 (0.1 gg/ml) 40 5
1 :100 0.2 2 (0.1 gg/ml) 40 5
1 250 0.08 0.8 (0.1 gg/ml) 40 5
1 500 0.04 4 (0.01 gg/ml) 40 5
1 1000 0.02 2 (0.01 4g/ml) 40 5
1 2000 0.01 1 (0. 0 1 gg/ml) 40 5
1 2500 0.008 0.8 (0.01 gg/ml) 40 5
Kontrolle 20 2.5

g) Analyse der Spaltprodukte durch N-terminale Sequenzierung

Die Verdauansaetze mit einem Massenverhaeltnis Trypsin : rh-**proNGF** von a) 1 40; b) 1: 100
und c) 1 : 250 wurden durch N-terminale Sequenzierung naeher untersucht. In der Bande bei
13 kDa fanden sich mehrere Spezies (Figur 8).

Zur Gewinnung von maturem rh-ss-NGF aus rh-**proNGF** im praeparativen Massstab wurden 1.3
mg rh-**proNGF** (in 50 mM Tris/HCI pH 8.0; Konzentration 0.46 mg/rnl) im Masseverhaeltnis
1:250 (Trypsin : rh-**proNGF**) mit Trypsin versetzt. Der Ansatz wurde 30 min auf Eis inkubiert.

Tabelle 4
Spezies ECro-Wert [pg/ml]
rh-ss-NGF 110
rh-ss-NGF, hergestellt durch limitierte Proteolyse von rh-**proNGF** 171 1

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PCT/EP99/07613

CLMDE Verfahren zur Herstelluncy eines biolocrisch aktiven ss-NGF aus seiner inaktiven schwer
C C
loeslichen Proform, erhaeltlich nach rekombinanter Herstellung in Prokaryonten, dadurch
C
gekennzeichnet, dass **proNGF** in seiner inaktiven schwer
loeslichen Form mit einer
Loesunor eines Denaturierunasmittels in einer denaturierenden Konzentration Creloest
13 C Z@
wird, anschliessend unter Erhalt der Loeslichkeit in eine nicht oder schwach **denaturie**-
rende Loesuncy ueberfuehrt wird und dabei denaturierter **proNGF**
eine biologisch aktive
Konformation annimmt, die durch die im natuerlichen ss-NGF vorliegenden Disulfid-
bruecken bestimmt ist, und anschliessend die Pro-Sequenz abgrespalten wird, wobei akti-
ver ss-NGF erhalten wird, der isoliert werden kann.

7 Verfahren nach den Anspruechen 1 bis 6, dadurch gekennzeichnet, dass als **Denaturie**-
rungsmittel Guanidiniumhydrochlorid oder Harnstoff verwendet wird.
Fig. 1
T7 Term inator (260-21 3)
am p r (61 05-5245) Bam H 1 (320)
.',@**proNGF** (996-328)
Nde 1 (996)
rbs (1010-1005)
ac Operator (1 065-1 041)
pET11a-**proNGF** 7 Promoter (1084)
6313 bp
ColE1 ori (4487) lacl (1471-2553)
ERSMZBLATT (REGEL 26)
Fig. 2
1 p s
2/11
Fig. 2a
pH-Optimierung
45
o@' 30
(D o
5 5.5 6 6.5 7 7.5 8 8.5 9 9.5 10
PH-Wert
Fig. 2b
Arginin-Optimlerung

Variation der GSHuKonzent ion (c(GSSG) 1 mM)
35 -
< 10
0 2 4 6 8 10 12 14 16 18 20
GSH-Konzentration [mM]
Fig. 2d
Variation der GSSGmKonzentration (c(GSH) 5 mM)
40 -
35 -
30 -
o@O
20
(D
m
U) 1 5
:3
< io
5
0 T
0 2 4 6 8 10 12 14 16 18 20
GSSG-Konzentration [mM]
4/11
ERSATZBLATT(REGEL26)
Fig. 2e
Optimierung der GdmCI-Konzentration
-
-
-
< lo
0 50 100 150 200 250 300 350 400 450 500
GdmCI-Konzentration [mM]
Fig. 2f
Optimierung der Proteinkonzentration
-
cn
Z
< 15
0 100 200 300 400 500
Proteinkonzentration [pg/ml]
5/11
ERSATZBLATT(REGEL26)
Fig. 3
Elutionsprofil der Reinigung von rh-**proNGF** an Poros 20 HS
245
UV1 280 nm mAU
195
..... Conc %B
:D
< Cond%
E
145
E
CD
OD
NN..@ 95
45 -
-5 .....
15 25 35 45 55 65 75 85 95
Elutionsvolumen [ml]

100 kDa
66 kDa

20 kDa@+',
10 kDa
7 /11
Fig. 5
UV-Spektrum rh-**proNGF**
0.4 -
0.35 -- **proNGF**-Monomer:
0.3 - 11 Phe
4 Trp
0.25 - 2 Tyr
0.2
0.15
0.1
0.05
240 250 260 270 280 290 300 310 320 330 340
Wellenlaenge [nm]
8/11
ERSATZBLATT(REGEL26)
Fig. 6
proNGF 50 pl UV VIS 2
WVL@280 nm
0
E.1 0.0 -
5.941 min
0-
0.0 1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0
min
9/11
ERSATZBLATT(REGEL26)
- WO 00/22119 PCT/EP99/07613
Fig. 7
0,10 -
E
c 0,05 -
CD
c\I
r+*i
.O
< 0.00 -,
-os05 --1 1 1 1
0 1 0 15 20 25 30
- Zeit [min]
10/11
ERSATZBLATT(REGEL26)
Fig. 8
M 1 2 3 4 5 6 7 8 9 10
50 kDa ....
40 kD
30 kDa
20 kD
10 kDa
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<110> Rudolph, Prof. Dr. Rainer
<120> Verfahren zur Gewinnung von aktivem beta-NGF
<130> P11700
<140>
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<150> EP 98119077.0
<151> 1998 09
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4 0 4 5
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Leu Arg Ser Pro Arg Val Leu Phe Ser Thr Gln Pro Pro Arg Glu Ala
70 75 8 0
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Arg Gly Glu Phe Ser Val Cys Asp Ser Val Ser Val Trp Val Gly Asp
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145 150 155 160
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Ser Lys His Trp Asn Ser Tyr Cys Thr Thr Thr His Thr Phe Val Lys
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Ala Leu Thr Met Asp Gly Lys Gln Ala Ala Trp Arg Phe Ile Arg Ile
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Asp Thr Ala Cys Val Cys Val Leu Ser Arg Lys Ala Val Arg
210 215 220
INTERNATIONAL SEARCH REPORT interr, inal Application Na
PCT/EP 99/07613
A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 CO7 K14/48
Aocarcling to International Patent Classification (IPC) or to both
national classdication and IPC
B. FIELDSSEARCHED
Minimum daaumentation searched (classdiaation system followed by
classification Symbols)
IPC 7 C12N CO7K
Dacumentation searched other than minimum documentation to the extent
that such documents ans included in the fields searohed
Electronic data base Gonsulted during the international search (name of
data base and, where practiral, search terms used)
C. DOCUMENTS CONSIDERED TO BE RELEVANT
Gategory Citation of document, with indication, where appropriate, of
the relevant passages Relevant to claim Na.
x EP 0 544 293 A (BOEHRINGER MANNHEIM GMBH 1-7
(DE); LANG; BARTKE; NAUJOKS; RUDOLPH;
STERN) 2 June 1993 (1993 02)
cited in the application
abstract
plage 3, line 45 -page 4, line 34
page 4, line 54 -page 5, line 23
page 6, line 24 -page 7, line 1
x EP 0 786 520 A (BOEHRINGER MANNHEIM GMBH 1-7
(DE); LANG; BARTKE; NAUJOKS; RUDOLPH;
STERN) 30 July 1997 (1997 30)
abstract
page 3, line 9 -page 4, line 23
page 5, line 16 -page 7, line 29
page 13, line 39,40
rther documents are listed in the continuation of box C.
Fu rv Patent family members are listed in annex.
Special categories of eited documents
r later dacument published after the international filing date
'A' doaument defining the general state of the art which is not or
filing date cannot be considered novel or cannot be considered t
document which may throw cloubts an priority claim 9 or involve an

of partiaular relevance; claimed invention
eitation or other special reason (as specified) cannot be considered to involve an inventive step when the
'0' dacum nt referring to an oral disolosure, use, exhibkion or dacument is combinecl with one or more other such docu-
el
other means ments, auch combination being obvious to a Person sIdlIed
'P' clocument published phor to the international filing date but in the art.
later than the priority date clairned W documentmemberoftheaamepatentfam ily
Date of the actual rompletion of the internabonal search Date of mailing of the international search report
11 February 2000 1 0403,00
Name and mailing addreas of the ISA Authorized offiaer
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo ni,
Fax: (+31-70) 340-301 6 Macchia, G
Forrn PCT/ISA/210 (second sheet) (July 1992)
page 1 of 2
fflERNATIONAL SEARCH REPORT Interk inal Application No
PCT/EP 99/07613
C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT
Category' CMtionofdocument,wfthindication,whereappropriatc,ofthercievant passages Relevant to claim No.
A SUTER U. ET AL.: `Two conserved domains in the NGF propeptide are necessary and sufficient for the biosynthesis of correctly processed and biologically active NGF
THE EMBO JOURNAL,
vol. 10, no. 9, 1991, pages 2395-2400,
XPO02095460
cited in the application
abstract
page 2399
A WO 97 28272 A (TECHNOLOGENE INC. (US);
SGARLATO G.D.) 7 August 1997 (1997 07)
page 27, line 24 -page 29, line 21
page 101, line 11 -page 106; example 6
Form PCTASA/210 (continuation of second sheet) (July 1992)
page 2 of 2
INTERNATIONAL SEALRCH REPORT
Intern, ial Application No
Information on patent lamily members PCT/EP 99/07613
Patent document Publication Patent family Publication
cited in search report date member(S) date
EP 0544293 A 02 1993 DE 4139000 A 03 1993
AT 158814 T 15 1997
DE 59208942 D 06 1997
EP 0786520 A 30 1997
jp 2637392 B 06 1997
jp 9023883 A 28 1997
jp 2611102 B 21 1997
jp 6327489 A 29 1994
EP 0786520 A 30 1997 DE 4139000 A 03 1993
AT 158814 T 15 1997

Inter 'onales Aktenzeiche
PC'i/EP 99/07613
X. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES
IPK 7 C12N15/12 CO7K14/48
Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK
B. RECHERCHIERTE GEBIETE
Recherchierter Mindestpruefstoff (Klassifikationssystenn und Klassifikationssymbole
IPK 7 C12N CO7K
Recherchierte aber nicht zum Mindestpruefstoff gehoerende Veroeffentlichungen, soweit diese unter die recherchierten Gebiete fallen
Waehrend der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)
C. ALS WESENTLICH ANGESEHENE UNTERLAGEN
Kategorie3 Bezeichnung der Veroeffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile Betr. Anspruch Nr.
X EP 0 544 293 A (BOEHRINGER MANNHEIM GMBH 1-7
(DE); LANG; BARTKE; NAUJOKS; RUDOLPH;
STERN) 2. Juni 1993 (1993 02)
in der Anmeldung erwaehnt
Zusammenfassung
Seite 3, Zeile 45 -Seite 4, Zeile 34
Seite 4, Zeile 54 -Seite 5, Zeile 23
Seite 6, Zeile 24 -Seite 7, Zeile 1
X EP 0 786 520 A (BOEHRINGER MANNHEIM GMBH 1-7
(DE); LANG; BARTKE; NAUJOKS; RUDOLPH;
STERN) 30. Juli 1997 (1997 30)
Zusammenfassung
Seite 3, Zeile 9 -Seite 4, Zeile 23
Seite 5, Zeile 16 -Seite 7, Zeile 29
Seite 13, Zeile 39,40
r71 WeitereVeoeffentlichungensindderFortsetzungvonFeld13zu Siehe Anhang Patentfamilie
A
L.J entnehmenr
Besondere Kategorien von angegebenen Veroeffentlichungen -r ire Veroeffentlichung, die nach dem internationalen Anmeldedatum
So%'eter d m Prioritaetsdatum veroeffentlicht worden ist und mit der
A' Veroeffentlichung, die den allgemeinen Stand der Technik definiert, e aber nicht als besonders bedeutsam anzusehen ist Anme dung nicht kollidiert, sondern nur zum Verstaendnis des der Erfindung zugrundeliegenden Prinzips oder der ihr zugrundeliegenden
'E' aelteres Dokument, das jedoch erst am oder nach dem internabonalen Theorie angegeben ist
Ammeicledatum veroeffentlicht worden ist 'X' Veroeffentlichung von besonderer Bedeutung; die beanspruchte Erfindung
oLu Veroeffentlichung, die geeignet ist, einen Prioritaetsanspruch zweifelhaft er- kann allein aufgrund dieser Veroeffentlichung nicht als neu oder auf
scheinen zu lassen, oder durch die das Veroeffentlichungsdatum einer erfinderischer Taetigkeit beruhend betrachtet werden
anderen im Recherchenbericht genannten Veroeffentlichung belegt werden
Yo Veroeffentlichung von besonderer Bedeutung; die beanspruchte Erfindung
soll oder die aus einem anderen besonderen Grund angegeben ist (wie kann nicht als auf erfinderischer Taetigkeit beruhend betrachtet ausgefuehrt) werden, wenn die Veroeffentlichung mit einer oder mehreren anderen

nach
denn beanspruchten Prioritaetsdatum veroeffentlicht worden ist qic

internationalen Recherche[redacted]richts

L8 ANSWER 19 OF 19 MEDLINE DUPLICATE 1

AB The complete mouse prepro-nerve growth factor (NGF) DNA was fused to the carboxyl terminus of the beta-galactosidase (lac-z) gene of Escherichia coli. Similarly, a genomic fragment encoding the human NGF comprising codons 11 to 106 (from a total of 118) was fused to the fifth codon of the amino terminus of beta-galactosidase. Both bacterial vectors produce high amounts of the chimeric proteins. After cell lysis most of the chimeric mouse **preproNGF** protein is insoluble and appears in the pellet, whereas the majority of the chimeric human beta-NGF remains in the supernatant. Purification of the fusion proteins from the soluble fraction was achieved by affinity chromatography to p-aminophenyl beta-D-thio-galactoside Sepharose. Yields of the purified chimeric proteins were increased threefold to fourfold by the addition of protease inhibitors in the lysis and chromatography buffers. Their antigenic similarity to the **preproNGF** and mouse beta-NGF was examined by their interaction to sera raised against synthetic peptides which reproduce sequences of the precursor protein and to sera directed against native and **denatured** mouse beta-NGF using enzyme-linked immunoabsorbent assay (ELISA) techniques. Antibodies to the peptide N2 (-163 to -139) interacted with high affinity with the chimeric mouse **preproNGF** protein. Antisera to native and **denatured** mouse beta-NGF interacted with both chimeric proteins but with a variable degree of affinity. These results provide direct evidence that certain antisera to mouse beta-NGF can cross-react with the human beta-NGF molecule.

corresponding restricti[redacted] enzymes, The plasmid pC8hB was derived by cloning [redacted] human BDNF coding sequences, including the entire prepro region, into the expression vector pCDM8 (discussedlsupra), This fragment was ligated to pDSRa2 (see published European patent application 90305433.6 EP0 Publication No, 0398753A2. incorporated herein by reference in its entirety, The plasmid pDSRa2 had been previously digested to make available the cloning sites 51-HindIII and 31-Sall for ligation of the human BDNF containing fragment. The resulting plasmid was designated pDSRa2(BDNF).

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=> s nerve growth factord hist
L6            0 NERVE GROWTH FACTORD HIST

=> d hist

     (FILE 'HOME' ENTERED AT 09:25:06 ON 17 MAR 2003)

     FILE 'MEDLINE, BIOSIS, USPATFULL, PCTFULL' ENTERED AT 09:25:38 ON 17 MAR
     2003
L1         36485 S NERVE GROWTH FACTOR
L2            82 S PRONGF OR PREPRONGF
L3            36 S L2 AND (NATUR##### OR DENATUR##### OR RENATUR#####)
L4            34 DUPLICATE REMOVE L3    (2 DUPLICATES REMOVED)
L5            22 S L4 AND ARGININE
L6             0 S NERVE GROWTH FACTORD HIST

=> s l2 and (denatur##### or renatur#####)
L7           20 L2 AND (DENATUR##### OR RENATUR#####)

=> duplicate remove
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DUPLICATE PREFERENCE IS 'MEDLINE, BIOSIS, USPATFULL, PCTFULL'
KEEP DUPLICATES FROM MORE THAN ONE FILE?  Y/(N):n
PROCESSING COMPLETED FOR L7
L8            19 DUPLICATE REMOVE L7 (1 DUPLICATE REMOVED)

=> s l8 and arginine
L9            15 L8 AND ARGININE

=> d 1-15

L9   ANSWER 1 OF 15  USPATFULL
AN     2002:251935  USPATFULL
TI     Purification of NGF
IN     Burton, Louis E., San Mateo, CA, UNITED STATES
       Schmelzer, Charles H., Burlingame, CA, UNITED STATES
       Beck, Joanne T., Westlake Village, CA, UNITED STATES
PI     US 2002137893      A1   20020926
AI     US 2002-72681      A1   20020208 (10)
RLI    Continuation of Ser. No. US 2000-675503, filed on 29 Sep 2000, GRANTED,
       Pat. No. US 6423831 Continuation of Ser. No. US 1999-363573, filed on 29
       Jul 1999, GRANTED, Pat. No. US 6184360 Continuation of Ser. No. US
       1997-970865, filed on 14 Nov 1997, GRANTED, Pat. No. US 6005081
PRAI   US 1996-30838P      19961115 (60)
       US 1997-47855P      19970528 (60)

       [illegible]
NCT    NCLM:  [illegible]
       NCLS:  [illegible]
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CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 2 OF 15 USPATFULL
AN 2002:181791 USPATFULL
TI Isolation of neurotrophins from a mixture containing other proteins and neurotrophin variants using hydrophobic interaction chromatography
IN Burton, Louis E., San Mateo, CA, United States
Schmelzer, Charles H., Burlingame, CA, United States
Beck, Joanne T., Westlake Village, CA, United States
PA Genentech, Inc., So. San Francisco, CA, United States (U.S. corporation)
PI US 6423831 B1 20020723
AI US 2000-675503 20000929 (9)
RLI Continuation of Ser. No. US 1999-363573, filed on 29 Jul 1999, now patented, Pat. No. US 6184360 Continuation of Ser. No. US 1997-970865, filed on 14 Nov 1997, now patented, Pat. No. US 6005081
PRAI US 1997-47855P 19970529 (60)
US 1996-30838P 19961115 (60)
DT Utility
FS GRANTED
LN.CNT 2348
INCL INCLM: 530/399.000
INCLS: 530/324.000; 530/350.000; 530/412.000; 530/416.000; 530/417.000; 435/069.100; 435/069.400; 435/070.100; 435/071.100
NCL NCLM: 530/399.000
NCLS: 435/069.100, 435/069.400; 435/070.100; 435/071.100; 530/324.000; 530/350.000; 530/412.000; 530/416.000; 530/417.000
IC [7]
ICM: C07K003-14
ICS: C12P021-06
EXF 530/399; 530/324; 530/350; 530/412; 530/416; 530/417; 435/69.1; 435/69.4; 435/70.1; 435/71.1
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 3 OF 15 USPATFULL
AN 2002:85534 USPATFULL
TI NOVEL NEUROTROPHIC FACTOR
IN ROSENTHAL, ARNON, PACIFICA, CA, UNITED STATES
PI US 2002045576 A1 20020418
US 6506728 B2 20030114
AI US 1995-450842 A1 19950526 (8)
RLI Division of Ser. No. US 1995-426419, filed on 19 Apr 1995, ABANDONED Continuation of Ser. No. US 1993-30013, filed on 22 Mar 1993, ABANDONED A 371 of International Ser. No. WO 1991-US6950, filed on 24 Sep 1991, UNKNOWN Continuation-in-part of Ser. No. US 1991-648482, filed on 31 Jan 1991, ABANDONED Continuation-in-part of Ser. No. US 1990-587707, filed on 25 Sep 1990, GRANTED, Pat. No. US 5364769
DT Utility
FS APPLICATION
LN.CNT 2815
INCL INCLM: 514/012.000
INCLS: 514/002.000
NCL NCLM: 514/012.000
NCLS: 514/002.000
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ICS: A61K038-17
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 4 OF 15 USPATFULL
[illegible]
corporation)
PI US 6184360 B1 20010206
[illegible]

patented, Pat. No. US 6[redacted]081
PRAI US 1996-30838P 1996[redacted]15 (60)
US 1997-47855P 19970529 (60)
DT Utility
FS Granted
LN.CNT 2226
INCL INCLM: 530/399.000
INCLS: 530/324.000; 530/350.000; 530/412.000; 530/416.000; 530/417.000; 435/069.100; 435/069.400; 435/070.100; 435/071.100
NCL NCLM: 530/399.000
NCLS: 435/069.100; 435/069.400; 435/070.100; 435/071.100; 530/324.000; 530/350.000; 530/412.000; 530/416.000; 530/417.000
IC [7]
ICM: C07K003-14
ICS: C12P021-06
EXF 530/399; 530/324; 530/350; 530/412; 530/416; 530/417; 435/69.1; 435/69.4; 435/70.1; 435/71.1
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 5 OF 15 USPATFULL
AN 2001:7868 USPATFULL
TI Neuronal factor
IN Rosenthal, Arnon, Pacifica, CA, United States
Winslow, John W., El Granada, CA, United States
PA Genentech, Inc., So. San Francisco, CA, United States (U.S. corporation)
PI US 6174701 B1 20010116
AI US 1995-455741 19950531 (8)
RLI Continuation of Ser. No. US 1995-381030, filed on 31 Jan 1995 Continuation of Ser. No. US 1990-494024, filed on 15 Mar 1990, now abandoned Continuation-in-part of Ser. No. US 1989-449811, filed on 12 Dec 1989, now abandoned
DT Utility
FS Granted
LN.CNT 1480
INCL INCLM: 435/069.100
INCLS: 536/023.500; 435/320.100; 435/325.000; 435/352.000; 435/354.000; 435/357.000; 435/358.000; 435/364.000; 435/366.000; 435/367.000; 435/252.300; 435/252.330; 435/069.700; 435/069.800
NCL NCLM: 435/069.100
NCLS: 435/069.700; 435/069.800; 435/252.300; 435/252.330; 435/320.100; 435/325.000; 435/352.000; 435/354.000; 435/357.000; 435/358.000; 435/364.000; 435/366.000; 435/367.000; 536/023.500
IC [7]
ICM: C12N015-00
ICS: C12N005-02; C12P021-06; C07H021-04
EXF 435/6; 435/69.1; 435/69.8; 435/240.2; 435/252.3; 435/252.33; 435/320.1; 435/325; 435/352; 435/354; 435/357; 435/358; 435/364; 435/366; 435/367; 536/23.5; 536/24.3; 536/24.31; 536/24.33
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 6 OF 15 USPATFULL
AN 2000:31394 USPATFULL
TI Neurotrophic factor (NT-4)
IN Rosenthal, Arnon, Pacifica, CA, United States
PA Genentech, Inc., South San Francisco, CA, United States (U.S. corporation)
PI US 6037320 20000314
AI US 1997-928694 19970912 (8)
RLI Continuation of Ser. No. US 1995-451947, filed on 26 May 1995, now patented, Pat. No. US 5702906 which is a division of Ser. No. US

IC [7]
ICM: A61K038-18
ICS: C07K014-475
EXF 514/2; 514/12; 530/350
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 7 OF 15 USPATFULL
AN 1999:167121 USPATFULL
TI Purification of recombinant human neurotrophins
IN Burton, Louis E., San Mateo, CA, United States
Schmelzer, Charles H., Burlingame, CA, United States
Beck, Joanne T., Westlake Village, CA, United States
PA Genentech, Inc., South San Francisco, CA, United States (U.S. corporation)
PI US 6005081 19991221
AI US 1997-970865 19971114 (8)
PRAI US 1996-30838P 19961115 (60)
US 1997-47855P 19970529 (60)
DT Utility
FS Granted
LN.CNT 2397
INCL INCLM: 530/399.000
INCLS: 530/324.000; 530/350.000; 530/412.000; 530/416.000; 530/417.000; 435/069.100; 435/069.400; 435/070.100; 435/071.100
NCL NCLM: 530/399.000
NCLS: 435/069.100; 435/069.400; 435/070.100; 435/071.100; 530/324.000; 530/350.000; 530/412.000; 530/416.000; 530/417.000
IC [6]
ICM: C07K003-14
ICS: C12P021-06
EXF 530/399; 530/324; 530/350; 530/412; 530/416; 530/417; 435/69.1; 435/69.4; 435/70.1; 435/71.1
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 8 OF 15 USPATFULL
AN 1999:92541 USPATFULL
TI Protein expression system
IN Sgarlato, Gregory D., Los Gatos, CA, United States
PA Technologene, Inc., Los Gatos, CA, United States (U.S. corporation)
PI US 5935824 19990810
AI US 1996-595043 19960131 (8)
DT Utility
FS Granted
LN.CNT 5959
INCL INCLM: 435/069.700
INCLS: 435/069.800; 530/350.000; 536/023.400
NCL NCLM: 435/069.700
NCLS: 435/069.800; 530/350.000; 536/023.400
IC [6]
ICM: C07K019-00
ICS: C12N015-62
EXF 435/69.7; 435/69.8; 435/207; 435/68.1; 436/532; 436/828; 530/387.1; 530/350; 530/413; 530/812; 530/866; 530/867; 536/23.4; 536/23.2; 536/23.53; 536/23.7; 935/47
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 9 OF 15 USPATFULL
AN 1998:135007 USPATFULL
TI Neurotrophic factor
IN Rosenthal, Arnon, Pacifica, CA, United States

Abandoned which is a continuation of Ser. No. US 1991-641482, filed on 15 Jan 1991, now abandoned which is a continuation in part of Ser. No.

FS Granted
LN.CNT 2363
INCL INCLM: 514/012.000
INCLS: 514/002.000; 530/350.000; 530/395.000; 530/399.000; 530/402.000; 435/069.100
NCL NCLM: 514/012.000
NCLS: 435/069.100; 514/002.000; 530/350.000; 530/395.000; 530/399.000; 530/402.000
IC [6]
ICM: A61K038-18
ICS: C07K014-475
EXF 514/2; 514/12; 530/350; 530/395; 530/399; 530/402; 435/69.1
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 10 OF 15 USPATFULL
AN 97:123048 USPATFULL
TI Antibodies to neurotrophic factor-4 (NT-4)
IN Rosenthal, Arnon, Pacifica, CA, United States
PA Genentech, Inc., South San Francisco, CA, United States (U.S. corporation)
PI US 5702906 19971230
AI US 1995-451947 19950526 (8)
RLI Division of Ser. No. US 1995-426419, filed on 19 Apr 1995 which is a continuation of Ser. No. US 1993-30013, filed on 22 Mar 1993, now abandoned which is a continuation-in-part of Ser. No. US 1991-648482, filed on 31 Jan 1991, now abandoned which is a continuation-in-part of Ser. No. US 1990-587707, filed on 25 Sep 1990, now patented, Pat. No. US 5364769
DT Utility
FS Granted
LN.CNT 2046
INCL INCLM: 435/007.100
INCLS: 530/387.100; 530/387.900; 530/388.240; 530/413.000; 435/336.000; 435/236.000
NCL NCLM: 435/007.100
NCLS: 435/236.000; 435/336.000; 530/387.100; 530/387.900; 530/388.240; 530/413.000
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ICM: G01N033-53
ICS: C12N005-12; C07K016-22; C07K001-16
EXF 424/139.1; 424/141.1; 424/145.1; 424/9.1; 435/7.1; 435/336; 530/387.1; 530/387.9; 530/388.1; 530/388.15; 530/388.24; 530/389.1; 530/389.2; 530/391.3; 530/413
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 11 OF 15 USPATFULL
AN 94:99824 USPATFULL
TI Nucleic acid encoding neurotrophic factor four (NT-4), vectors, host cells and methods of production
IN Rosenthal, Arnon, Pacifica, CA, United States
PA Genentech, Inc., South San Francisco, CA, United States (U.S. corporation)
PI US 5364769 19941115
AI US 1990-587707 19900925 (7)
DT Utility
FS Granted
LN.CNT 1357
INCL INCLM: 435/069.100
INCLS: 435/069.400; 435/320.100; 435/240.100; 435/240.200; 536/023.500;
[illegible]
[illegible]
EXF [illegible]
435/240.1; 435/240.2
[illegible]

L9 ANSWER 12 OF 15 PCTFULL COPYRIGHT 2003 Univentio
AN 1997028272 PCTFULL ED 20020514
TIEN PROTEIN EXPRESSION SYSTEM
TIFR SYSTEME D'EXPRESSION DE PROTEINES
IN SGARLATO, Gregory, D.
PA TECHNOLOGENE INC.
LA English
DT Patent
PI WO 9728272 A1 19970807
DS W: AU CA JP AT BE CH DE DK ES FI FR GB GR IE IT LU MC NL PT SE
AI WO 1997-US1470 A 19970131
PRAI US 1996-8/595,043 19960131
ICM C12P021-00
ICS C12P021-06; C07K001-12; C07K001-22; C07K014-46; C07K014-195;
C07K016-00; C07K019-00; C07H021-04; C12N009-38

L9 ANSWER 13 OF 15 PCTFULL COPYRIGHT 2003 Univentio
AN 1993025684 PCTFULL ED 20020513
TIEN THERAPEUTIC AND DIAGNOSTIC METHODS BASED ON NEUROTROPHIN-4 EXPRESSION
TIFR PROCEDES THERAPEUTIQUE ET DIAGNOSTIQUE BASES SUR L'EXPRESSION DE LA
NEUROTROPHINE-4
IN IP, Nancy;
ALTAR, Charles, A.;
DISTEFANO, Peter;
VENTIMIGLIA, Roscann;
WIEGAND, Stanley;
WONG, Vivien;
YANCOPOULOS, George, D.
PA REGENERON PHARMACEUTICALS, INC.;
IP, Nancy;
ALTAR, Charles, A.;
DISTEFANO, Peter;
VENTIMIGLIA, Roseann;
WIEGAND, Stanley;
WONG, Vivien;
YANCOPOULOS, George, D.
LA English
DT Patent
PI WO 9325684 A1 19931223
DS W: AU BB BG BR BY CA CZ FI HU JP KR KZ LK MG MN MW NO NZ PL RO
RU SD SK UA US AT BE CH DE DK ES FR GB GR IE IT LU MC NL PT
SE BF BJ CF CG CI CM GA GN ML MR NE SN TD TG
AI WO 1993-US5672 A 19930611
PRAI US 1992-898,194 19920612
ICM C12N015-12
ICS C12Q001-68; C12P021-08; A61K037-02

L9 ANSWER 14 OF 15 PCTFULL COPYRIGHT 2003 Univentio
AN 1992020365 PCTFULL ED 20020513
TIEN THERAPEUTIC AND DIAGNOSTIC METHODS BASED ON NEUROTROPHIN-4 EXPRESSION
TIFR PROCEDES THERAPEUTIQUES ET DIAGNOSTIQUES BASEES SUR L'EXPRESSION DE
NEUROTROPHINE-4
IN HALLBOOK, Finn;
IBANEZ MOLINER, Carlos, Fernando;
PERSSON, Hakan, Bengt;
IP, Nancy;
YANCOPOULOS, George, D.
PA REGENERON PHARMACEUTICALS, INC.;
HALLBOOK, Finn;
IBANEZ MOLINER, Carlos, Fernando;

NO FI SE
AI WO 1992-US4266 A 19920521

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       US 1991-734,422          19910723
       US 1991-751,356          19910828
       US 1991-762,674          19910920
       US 1991-791,924          19911114
ICM    A61K037-02
ICS    A61K049-00; G01N033-50; G01N033-68; C07K039-00; C12N015-12;
       C12N015-79

L9     ANSWER 15 OF 15   PCTFULL   COPYRIGHT 2003 Univentio
AN     1992005254 PCTFULL  ED 20020513
TIEN   NOVEL NEUROTHROPHIC FACTOR
TIFR   NOUVEAU FACTEUR NEUROTROPHIQUE
IN     ROSENTHAL, Arnon
PA     GENENTECH, INC.;
       ROSENTHAL, Arnon
LA     English
DT     Patent
PI     WO 9205254           A1 19920402
DS     W:          AT AU BE CA CH DE DK ES FR GB GR IT JP LU NL SE US
AI     WO 1991-US6950       A  19910924
PRAI   US 1990-587,707         19900925
       US 1991-648,482         19910131
ICM    C12N015-12
ICS    C12Q001-68; C12P021-08; A61K037-02


=> d 3-5,7-9 hit

L9   ANSWER 3 OF 15  USPATFULL
DETD   [0030] NT-4 nucleic acid is RNA or DNA which encodes a NT-4 polypeptide
       or which hybridizes to such DNA and remains stably bound to it under
       stringent conditions and is greater than about 10 bases in length;
       provided, however, that such hybridizing nucleic acid is novel and
       unobvious over any prior art nucleic acid including that which encodes
       or is complementary to nucleic acid encoding NGF, BDNF, or NT-3.
       Stringent conditions are those which (1) employ low ionic strength and
       high temperature for washing, for example, 0.15 M NaCl/0.015 M sodium
       citrate/0.1% NaDodSO.sub.4 at 50.degree. C., or (2) use during
       hybridization a denaturing agent such as formamide, for
       example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1%
       Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6 5
       with 750 mM NaCl, 75 mM sodium citrate at 42.degree. C.
DETD   [0039] The third group of variants are those in which at least one amino
       acid residue in NT-4, and preferably only one, has been removed and a
       different residue inserted in its place. An example is the replacement
       of arginine and lysine by other amino acids to render the NT-4
       resistent to proteolysis by serine proteases, thereby creating a variant
       of NT-4 that is more stable The sites of greatest interest for
       substitutional mutagenesis include sites where the amino acids found in
       BDNF, NGF, NT-3, and NT-4 are substantially different in terms of side
       chain bulk, charge or hydrophobicity, but where there also is a high
       degree of homology at the selected site within various animal analogues
       of NGF, NT-3, and BDNF (e.g., among all the animal NGFs, all the animal
       NT-3s, and all the BDNFs). This analysis will highlight residues that
       may be involved in the differentiation of activity of the trophic
       factors, and therefore, variants at these sites may affect such
       activities. Examples of such sites in mature human NT-4, numbered from
       the N-terminal end, and exemplary substitutions include NT-4
       (G78.fwdarw.K, H, O or R) (SEQ ID NOS. 13, 14, 15, and 16, respectively)
       and NT-4 (P85.fwdarw.E, F, D, Y or W) (SEQ ID NOS. 17, 18,19, 20, and
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[illegible] conserved sites, are substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 1 under the heading [illegible]

exemplary substitutions in Table 1, or as further described below in reference to amino acid classes, are introduced and the products screened.

TABLE 1

Original Residue	Exemplary Substitutons	Preferred Substitutons
Ala (A)	val; leu; ile	val
Arg (R)	lys, gln; asn	lys
Asn (N)	gln, his, lys, arg	gln
Asp (D)	glu	glu
Cys (C)	ser	ser
Gln (Q)	asn	asn
Glu (E)	asp	asp
Gly (G)	pro	pro
His (H)	asn; gln; lys; arg;	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile, val; met, ala, phe	ile
Lys (K)	arg, gln, asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu, val; ile; ala	leu
Pro (P)	gly	gly
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Va (V)	ile; leu; met; phe; ala; norleucine	leu

DETD [0062] If the signal sequence is from another neurotrophic polypeptide, it may be the precursor sequence shown in FIG. 2 which extends from the initiating methionine (M) residue of NT-2, NT-3, or NGF up to the **arginine** (R) residue just before the first amino acid of the mature protein, or a consensus or combination sequence from any two or more of those precursors taking into account homologous regions of the precursors. The DNA for such precursor region is ligated in reading frame to DNA encoding the mature NT-4.

DETD [0088] Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of **arginine** residues requires that the reaction be performed in alkaline conditions because of the high pK.sub.a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the **arginine** epsilon-amino group.

DETD [0093] Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the o-amino groups of lysine, **arginine**, and histidine side chains (Creighton, Proteins: Structure and Molecular Properties, W. H. Freeman & Co., San Francisco, pp. 79-86), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. NT-4 also is covalently linked to nonproteinaceous polymers, e.g. polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the manner set forth in U.S. patent application No. 07/275,296 or U.S. Pat. Nos. 4,640,835, 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

DETD [0096] Therapeutic formulations of NT-4 are prepared for storage by mixing NT-4 having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers

[illegible] weight less than about 10 residues; polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins, hydrophilic polymers such as [illegible]

arginine

and other carbohydrates [redacted]luding glucose, mannose, or de[redacted]ins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or PEG.

DETD [0151] Aliquots of 200 .mu.l are taken from each 1 ml fraction collected, dialyzed against 1 M acetic acid, lyophilized, and redissolved in 30 .mu.l Laemmli SDS-PAGE sample buffer (Laemmli, 1970, Nature 7:680). Human .beta.-NGF is obtained in a similar manner. Following SDS-PAGE, the silver-stained gel indicates a single, prominently stained polypeptide of approximately 15 kD. A 3-ml pool of S-300 column eluted fractions corresponding to this SDS-PAGE analyzed region is made, and 1 ml (0.5 nmole) is submitted to N-terminal amino acid sequence analysis by Edman degradation performed on a prototype automated amino acid sequencer (Kohr, EP Pat. Pub. No. 257,735). N-terminal sequence analysis gives a single sequence starting with a glycine residue predicted by the tetrabasic cleavage sequence ending in an **arginine**, and predicted by the processing of **preproNGF** to mature .beta.-NGF.

L9 ANSWER 4 OF 15 USPATFULL

DETD MOPSO is 3-(N-Morpholino)-2-hydroxypropanesulfonic acid. HEPES is N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid. Reagent alcohol is 95 parts by volume (Specially **Denatured** Alcohol Formula 3A and 5 parts by volume isopropyl alcohol). MES is 2-(N-Morpholino)ethanesulfonic acid. UF/DF means ultrafiltration/diafiltration. TMAC is tetramethylammonium chloride. TEAC is tetraethylammonium chloride. NGF-120 means full-length of 120/120 nerve growth factor. NGF-118 means homodimeric mature NGF molecule of 118 residues. Oxidized NGF means NGF variant molecule, Metsulfoxide.sub.37, which is reported herein to be about 80% as biologically active as mature, native NGF. Isoasp NGF means NGF isomerized variant molecule, Asp93. Deamidated NGF means NGF having Asn45 converted to Asp45. RNGF means an NGF molecule with an extra **Arginine** residue at its N-terminus. CHO means Chinese hamster ovary cells.

DETD The concentration of neurotrophin in the buffered solution for solubilization must be such that the neurotrophin will be substantially solubilized and partially or fully reduced and **denatured**. Alternatively, the neurotrophin may be initially insoluble. The exact amount to employ will depend, e.g., on the concentrations and types of other ingredients in the buffered solution, particularly the type and amount of reducing agent, the type and amount of chaotropic agent, and the pH of the buffer. For example, the concentration of neurotrophin may be increased at least three-fold if the concentration of reducing agent, e.g., DTT, is concurrently increased, to maintain a ratio of DTT:neurotrophin of from about 3:1 to 10:1. It is desirable to produce a more concentrated solubilized protein solution prior to dilution refolding. Thus, the preferred concentration of neurotrophin is at least about 30 mg/mL, with a more preferred range of 30-50 mg per mL. For example, neurotrophin may be solubilized to a concentration of about 30-50 mg/mL in 5M to 7M urea, 10 mM DTT and diluted, for example, to about 1 mg/mL for folding.

DETD The degree of refolding that occurs upon this incubation is suitably determined by the RIA titer of the neurotrophin or by HPLC analysis with increasing RIA titer or correctly folded neurotrophin peak size directly correlating with increasing amounts of correctly folded, biologically active neurotrophin conformer present in the buffer. The incubation is carried out to maximize the yield of correctly folded neurotrophin conformer and the ratio of correctly folded neurotrophin conformer to misfolded neurotrophin conformer recovered, as determined by RIA or

denaturing

mammalian cell culture. For example, as was determined herein, rhNGF expressing CHO cell culture contained incorrectly proteolytically

precursor NGF sequences. Also found in the mammalian cell culture medium were glycosylated NGF and glycosylated forms of the incorrectly proteolytically processed variants. Undesirable glycosylated forms, which in the case of NGF can be seen as a higher molecular weight species (+2000 kD), could generate an unwanted antigenic response in a patient and contribute to poor product quality or activity. HIC effectively separated hydrophobic variants, primarily N-terminal-proteolytically-misprocessed variants, including glycosylated forms, from rhNGF. As shown in the examples, the precursor-sequence-containing and clipped precursor sequence NGF and the glycosylated forms of both NGF and the precursor-sequence-containing NGF eluted in the leading edge of the NGF peak during phenyl-HIC. Thus, a rhNGF composition could be obtained that was substantially free of these species, and that was particularly suited for a subsequent step such as high performance cation-exchange chromatography. HIC is applicable to other neurotrophins, as well as NGF, regardless of source. For example, HIC is useful to separate NGF monomers from dimers, either homo- or hetero-dimers depending on the monomer forms present, as well as distinguish dimer forms which also differ in hydrophobicity, that are obtained after in vitro refolding or when produced and secreted from mammalian cells. A preferred source of neurotrophin mixtures for use with HIC is mammalian cell culture, more preferably CHO cell culture. The culture is preferably subjected to at least one prior purification step as discussed herein. HIC is particularly effective in separating misprocessed glycosylated variant(s) from the native recombinant neurotrophin. In the case of rhNGF, the glycosylated and **preproNGF** forms are less hydrophobic than native NGF, thereby eluting before native NGF. Misfolded forms of neurotrophins (when bacterially produced) are also more hydrophobic, eluting earlier than the native neurotrophin.

DETD The carrier suitably contains minor amounts of additives such as substances that enhance isotonicity and chemical stability. Such materials are non-toxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, succinate, acetic acid, and other organic acids or their salts; antioxidants such as ascorbic acid; low molecular weight (less than about ten residues) polypeptides, e.g., polyarginine or tripeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids, such as glycine, glutamic acid, aspartic acid, or **arginine**; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, trehalose, glucose, mannose, or dextrins; chelating agents such as EDTA, sugar alcohols such as mannitol or sorbitol; counter-ions such as sodium; and/or nonionic surfactants such as polysorbates, poloxamers, or PEG. The final preparation may be a liquid or lyophilized solid.

DETD The microfiltrate was adjusted to 1M NaCl and applied to a Silica Gel Column equilibrated in 1M NaCl, 25mM MOPSO, pH 7. The column was washed with 1M NaCl, 25 mM MOPSO, pH7. Suitable pH range is about pH 6 to 8, with a preferred pH of 7. The column was then washed with 25 mM MOPSO, pH 7. A low conductivity wash removes host cell proteins. Bound NGF was eluted with 50 mM MOPSO, 0.5 MT MAC, 20% reagent anhydrous grade alcohol (94-96% Specially **Denatured** alcohol formula 3A (5 volumes of methanol and 100 volumes of 200 proof ethanol) and 4-6% isopropanol). Other alcohols can be used such as 20% propanol, 20% isopropanol and 20% methanol. As used herein, "alcohols" and "alcoholic solvents" are meant in the sense of the commonly used terminology for alcohol, preferably alcohols with 1 to 10 carbon atoms, more preferably methanol, ethanol, iso-propanol, n-propanol, or t-butanol, and most preferably ethanol or iso-propanol. Such alcohols are solvents that, when added to aqueous solution, increase the hydrophobicity of the solution by decreasing

can range from 0.1 to 1 M. With the range 0.3 to 0.7 M being more preferred. The amount of TMAC used to elute NGF is a function of pH and

the preferred pH was 7, [redacted]ch allows very minimal adjustm[redacted] of the pooled fractions prior to loading onto the next column. The upper pH limit is determined by the pH necessary to load the next column, and the lower limit by that useful to elute NGF efficiently.

DETD The SP-Sepharose HP effectively removed variants present in the HIC pool. The R120 form has an additional **arginine** residue at the N-terminus of NGF; usually the N-terminal amino acid sequence of rhNGF is SSSHP, but R120 has an N-terminal sequence of RSSSHP. Thus the R120 form is more basic than mature NGF and was separated by SP-SHP. It also has lower bioactivity, probably related to the fact that the NGF N-terminal is necessary for receptor (trkA) binding. The oxidized NGF form is a mono-oxidized form having the methionine at position 37 oxidized, yielding a more acidic form that elutes on the leading edge of the NGF peak. The isoasp form contains a modification of the aspartic acid at amino acid 93. The isoasp form is slightly more basic and thus binds slightly tighter to the SP-Sepharose HP resin. NGF species containing isoAsp93 eluted in the trailing edge of the elution peak. Deamidation occurs at asparagine residues, typically at asparagine at position 45. NGF containing deamidated Asn, which yields an Asp at position 45, is slightly more acidic, appearing at the leading edge of the elution peak.

DETD NT-4/5 was isolated from the inclusion bodies as follows. The inclusion body pellets were suspended in 20 mM Tris, pH 8, 6M Urea, 25 mM DTT (10 ml buffer/gram inclusion body) using a turrax at medium speed for about 10 min. The suspension was stirred for 40 min at 2-8.degree. C. and centrifuged in a Sorvall RC3B at 5000 rpm for about 45 min. PEI (poly-ethylene-imine) was added to 0.1% in the supernatant, which was stirred at 2-8.degree. C. for 30 minutes. The PEI precipitates nucleic acid and other acidic-charged molecules. The mixture was centrifuged in a Sorvall RC3B at 5000 rpm for about 45 minutes. The PEI supernatant was loaded onto a DEFF Sepharose Fast Flow column (10 cm.times.14 cm; DEFF is a diethyl aminoethyl resin) equilibrated in 0.02 M Tris, 6M Urea, 10 mM DTT, pH 8. An equivalent of 1 kg of solubilized refractile bodies was loaded onto the DEFF column. Since reduced and **denatured** NT-4/5 does not bind to the DEFF resin, the flow through pool containing NT-4/5 and 6M urea, was collected (FIG. 6) and the pH of the pool was lowered to 5.0 with acetic acid. The pH-adjusted DEFF flow through pool was loaded onto a S-Sepharose Fast Flow column (S refers to the SO3 functional group on the resin) equilibrated in 20 mM acetate, pH 5, containing 6M urea, under which conditions NT-4/5 binds to the resin. After loading, the S-Sepharose Fast Flow column was washed with several column volumes of equilibration buffer. The bound NT-4/5 was eluted with 0.5 M NaCl, 20 mM sodium acetate, 6M urea, pH 5 (FIG. 7). The 0.5 M NaCl SSFF pool was dialyzed overnight against 20 mM Tris, 0.14 M NaCl, pH 8, conditions that allow NT-4/5 to refold albeit incorrectly. The misfolded rhNT-4/5 molecules aggregated to form a precipitate.

L9 ANSWER 5 OF 15 USPATFULL

DETD NF nucleic acid is defined as RNA or DNA which encodes a NF polypeptide or which hybridizes to such DNA and remains stably bound to it under stringent conditions and is greater than about 10 bases in length, provided, however, that such hybridizing nuclic acid is novel and unobvious over any prior art nucleic acid including that which encodes or is complementary to nucleic acid encoding BDNF or NGF. Stringent conditions are those which (1) employ low ionic strength and high temperature for washing, for example, 0.15 M Nacl/0.015 M sodium citrate/0.1% NaDodSO.sub.4 at 50.degree. C., or (2) use during washing a **denaturing** agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM

[redacted] **arginine** [redacted]
render the NF resistent to proteolysis by serine porteases, thereby
crating a more stable NF analogue. The sites of greatest interest for

bulk, charge or hydrophobicity, but where there also is a high degree of homology at the selected site within various animal analogues of NGF and BDNF (e.g., among all the animal NGFs on the one hand and all the BDNFs on the other). This analysis will highlight residues that may be involved in the differentiation of activity of the trophic factors, and therefore, variants at these sites may affect such activities. Examples of such NF sites, numbered from the mature N-terminal end, and exemplary substitutions include NF (N.sub.85.fwdarw.K, H, Q or R) and NF (D.sub.72.fwdarw.E, F, P, Y or W). Other sites of interest are those in which the residues are identical among all animal species' BDNF, NGF and NF, this degree of conformation suggesting importance in achieving biological activity common to all three factors. These sites, especially those falling within a sequence of at least 3 other identically converved sites, are substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 1 under the heading of preferred substitutions. If such substitutions result in a change in biological acitivyt, then more substantial changes, denominated exemplary substitutions in Table 1, or as further described below in reference to amino acid classes, are introduced and the products screened.

DETD Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of **arginine** residues requires that the reaction be performed in alkaline conditions because of the high pK.sub.a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the **arginine** epsilon-amino group.

DETD Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the .alpha.-amino groups of lysine, **arginine**, and histidine side chains (T. E. Creighton, Proteins: Structure and Molecular Properties, W. H. Freeman & Co., San Francisco, pp. 79-86 [1983]), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. NF also is covalently linked to nonproteinaceous polymers, e.g. polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the manner set forth in U.S. Ser. No. 07/275,296 or U.S. Pat. Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

DETD Therapeutic formulations of NF are prepared for storage by mixing NF having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences, supra, in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone, amino acids such as glycine, glutamine, asparagine, **arginine** or lysine; monosaccharides, disaccharides and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or PEG.

DETD Aliquots of 200 .mu.l were taken from each 1 ml fraction collected, dialyzed against 1 M acetic acid, lyophilized, and redissolved in 30 .mu.l Laemmli SDS-PAGE sample buffer (Laemmli, Nature, 227: 680-685 (1970)). Human .beta.-NGF was obtained in a similar manner. Following SDS-PAGE, the silver-stained gel indicated a single, prominently stained polypeptide of approximately 15 kD. A 3 ml pool of S-300 column eluted

[illegible] amino acid residues [illegible] a single sequence starting with a tyrosine residue predicted by the tetrabasic cleavage sequence starting with a tyrosine residue predicted by the tetrabasic cleavage sequence [illegible] of preproNGF [illegible]

L9 ANSWER 7 OF 15 USPATFULL

DETD MOPSO is 3-(N-Morpholino)-2-hydroxypropanesulfonic acid. HEPES is N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid. Reagent alcohol is 95 parts by volume (Specially **Denatured** Alcohol Formula 3A and 5 parts by volume isopropyl alcohol). MES is 2-(N-Morpholino)ethanesulfonic acid. UF/DF means ultrafiltration/diafiltration. TMAC is tetramethylammonium chloride. TEAC is tetraethylammonium chloride. NGF-120 means full-length of 120/120 nerve growth factor. NGF-118 means homodimeric mature NGF molecule of 118 residues. Oxidized NGF means NGF variant molecule, Metsulfoxide.sub.37, which is reported herein to be about 80% as biologically active as mature, native NGF. Isoasp NGF means NGF isomerized variant molecule, Asp93. Deamidated NGF means NGF having Asn45 converted to Asp45. RNGF means an NGF molecule with an extra **Arginine** residue at its N-terminus. CHO means Chinese hamster ovary cells.

DETD The concentration of neurotrophin in the buffered solution for solubilization must be such that the neurotrophin will be substantially solubilized and partially or fully reduced and **denatured**. Alternatively, the neurotrophin may be initially insoluble. The exact amount to employ will depend, e.g., on the concentrations and types of other ingredients in the buffered solution, particularly the type and amount of reducing agent, the type and amount of chaotropic agent, and the pH of the buffer. For example, the concentration of neurotrophin may be increased at least three-fold if the concentration of reducing agent, e.g., DTT, is concurrently increased, to maintain a ratio of DTT:neurotrophin of from about 3:1 to 10:1. It is desirable to produce a more concentrated solubilized protein solution prior to dilution refolding. Thus, the preferred concentration of neurotrophin is at least about 30 mg/mL, with a more preferred range of 30-50 mg per mL. For example, neurotrophin may be solubilized to a concentration of about 30-50 mg/mL in 5M to 7M urea, 10 mM DTT and diluted, for example, to about 1 mg/mL for folding.

DETD The degree of refolding that occurs upon this incubation is suitably determined by the RIA titer of the neurotrophin or by HPLC analysis with increasing RIA titer or correctly folded neurotrophin peak size directly correlating with increasing amounts of correctly folded, biologically active neurotrophin conformer present in the buffer. The incubation is carried out to maximize the yield of correctly folded neurotrophin conformer and the ratio of correctly folded neurotrophin conformer to misfolded neurotrophin conformer recovered, as determined by RIA or HPLC, and to minimize the yield of multimeric, associated neurotrophin as determined by mass balance. Alternatively, the species can be determined via the methods provided below and in the Examples. Guanidine is a preferred **denaturing** agent for refolding.

DETD HIC was useful for separation of neurotrophins from their variants in mammalian cell culture. For example, as was determined herein, rhNGF-expressing-CHO cell culture contained incorrectly proteolytically processed variants, such as those in which a partial precursor sequence is present, e.g., precursor NGF, hybrid precursor NGF, and clipped precursor NGF sequences. Also found in the mammalian cell culture medium were glycosylated NGF and glycosylated forms of the incorrectly proteolytically processed variants. Undesirable glycosylated forms, which in the case of NGF can be seen as a higher molecular weight species (+2000 kD), could generate an unwanted antigenic response in a patient and contribute to poor product quality or activity. HIC effectively separated hydrophobic variants, primarily N-terminal-proteolytically-misprocessed variants, including glycosylated forms from rhNGF. As shown in the examples, the precursor sequence

[illegible] high performance cation exchange chromatography. HIC is applicable to other neurotrophins, as well as NGF, regardless of source. For example,

distinguish dimer forms which also differ in hydrophobicity that are obtained after in vitro refolding or when produced and secreted from mammalian cells. A preferred source of neurotrophin mixtures for use with HIC is mammalian cell culture, more preferably CHO cell culture. The culture is preferably subjected to at least one prior purification step as discussed herein. HIC is particularly effective in separating misprocessed glycosylated variant(s) from the native recombinant neurotrophin. In the case of rhNGF, the glycosylated and **preproNGF** forms are less hydrophobic than native NGF, thereby eluting before native NGF. Misfolded forms of neurotrophins (when bacterially produced) are also more hydrophobic, eluting earlier than the native neurotrophin.

DETD The carrier suitably contains minor amounts of additives such as substances that enhance isotonicity and chemical stability. Such materials are non-toxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, succinate, acetic acid, and other organic acids or their salts; antioxidants such as ascorbic acid; low molecular weight (less than about ten residues) polypeptides, e.g., polyarginine or tripeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids, such as glycine, glutamic acid, aspartic acid, or **arginine**; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, trehalose, glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; counter-ions such as sodium; and/or nonionic surfactants such as polysorbates, poloxamers, or PEG. The final preparation may be a liquid or lyophilized solid.

DETD The microfiltrate was adjusted to 1M NaCl and applied to a Silica Gel Column equilibrated in 1M NaCl, 25 mM MOPSO, pH 7. The column was washed with 1M NaCl, 25 mM MOPSO, pH7. Suitable pH range is about pH 6 to 8, with a preferred pH of 7. The column was then washed with 25 mM MOPSO, pH 7. A low conductivity wash removes host cell proteins. Bound NGF was eluted with 50 mM MOPSO, 0.5 M TMAC, 20% reagent anhydrous grade alcohol (94-96% Specially **Denatured** alcohol formula 3A (5 volumes of methanol and 100 volumes of 200 proof ethanol) and 4-6% isopropanol). Other alcohols can be used such as 20% propanol, 20% isopropanol and 20% methanol. As used herein, "alcohols" and "alcoholic solvents" are meant in the sense of the commonly used terminology for alcohol, preferably alcohols with 1 to 10 carbon atoms, more preferably methanol, ethanol, iso-propanol, n-propanol, or t-butanol, and most preferably ethanol or iso-propanol. Such alcohols are solvents that, when added to aqueous solution, increase the hydrophobicity of the solution by decreasing solution polarity. Ethanol is most preferred. The lower limit of alcohol is whatever percentage that elutes and the upper limit is set by the need to avoid protein **denaturation**. The solvent is preferably 5% to 25%, more preferably 5 to 20%, even more preferably 5 to 15%. TMAC is tetramethyl ammonium chloride, which is present to elute NGF. TMAC can range from 0.1 to 1 M. With the range 0.3 to 0.7 M being more preferred. The amount of TMAC used to elute NGF is a function of pH and alcohol concentration. The lower the pH the less amounts of alcohol and TMAC is required. The pH can be between about pH 4 to 8. In this example the preferred pH was 7, which allows very minimal adjustment of the pooled fractions prior to loading onto the next column. The upper pH limit is determined by the pH necessary to load the next column, and the lower limit by that useful to elute NGF efficiently.

DETD The SP-Sepharose HP effectively removed variants present in the HIC pool. The R120 form has an additional **arginine** residue at the N-terminus of NGF; usually the N-terminal amino acid sequence of rhNGF is SSSHP, but R120 has an N-terminal sequence of RSSSHP. Thus the R120 form is more basic than mature NGF and was separated by SP SHP. It also

[illegible] NGF peak. The isoasp form contains a modification of the aspartic acid at amino acid 93. The isoasp form is slightly more basic and thus binds slightly tighter to the SP Sepharose HP resin. NGF species [illegible]

position 45. NGF contain[redacted] deamidated Asn, which yields [redacted]Asp at position 45, is slightly more acidic, appearing at the leading edge of the elution peak.

DETD NT-4/5 was isolated from the inclusion bodies as follows. The inclusion body pellets were suspended in 20 mM Tris, pH 8, 6M Urea, 25 mM DTT (10 ml buffer/gram inclusion body) using a turrax at medium speed for about 10 min. The suspension was stirred for 40 min at 2-8.degree. C. and centrifuged in a Sorvall RC3B at 5000 rpm for about 45 min. PEI (poly-ethylene-imine) was added to 0.1% in the supernatant, which was stirred at 2-8.degree. C. for 30 minutes. The PEI precipitates nucleic acid and other acidic-charged molecules. The mixture was centrifuged in a Sorvall RC3B at 5000 rpm for about 45 minutes. The PEI supernatant was loaded onto a DEFF Sepharose Fast Flow column (10 cm.times.14 cm; DEFF is a diethyl aminoethyl resin) equilibrated in 0.02 M Tris, 6M Urea, 10 mM DTT, pH 8. An equivalent of 1 kg of solubilized refractile bodies was loaded onto the DEFF column. Since reduced and **denatured** NT-4/5 does not bind to the DEFF resin, the flow through pool containing NT-4/5 and 6M urea, was collected (FIG. 6) and the pH of the pool was lowered to 5.0 with acetic acid. The pH-adjusted DEFF flow through pool was loaded onto a S-Sepharose Fast Flow column (S refers to the SO3 functional group on the resin) equilibrated in 20 mM acetate, pH 5, containing 6M urea, under which conditions NT-4/5 binds to the resin. After loading, the S-Sepharose Fast Flow column was washed with several column volumes of equilibration buffer. The bound NT-4/5 was eluted with 0.5 M NaCl, 20 mM sodium acetate, 6M urea, pH 5 (FIG. 7). The 0.5 M NaCl SSFF pool was dialyzed overnight against 20 mM Tris, 0.14 M NaCl, pH 8, conditions that allow NT-4/5 to refold albeit incorrectly. The misfolded rhNT-4/5 molecules aggregated to form a precipitate.

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SUMM Early attempts at the expression of foreign proteins in E. coli utilized the bacterial .beta.-galactosidase (.beta.-gal) protein as the fusion partner. Many of the .beta.-gal fusion proteins were insoluble and were found in inclusion bodies [Itakura, K. et al., supra; Young, R. A. and Davis, R. W., Proc. Natl. Acad. Sci. USA 80:1194 (1983); Stanley, K. K. and Luzio, J. P., EMBO J. 31:429 (1984)]. In some cases, active fusion protein was recovered from the inclusion bodies by solubilization with **denaturing** reagents [Martson, A. O, Biochem. J. 240:1 (1986)]. In other cases, the fusion protein could not be recovered in an active form following **denaturation**, presumably due to an inability of the **denatured** protein to correctly refold upon **renaturation**.

SUMM Other means of purifying fusion proteins include the poly-**arginine** system, in which the fusion protein is selectively purified on a cation exchange resin [Sassenfeld, H. M. and Brewer, S. J. BioTechnology 2:76 (1984); U.S. Pat. No. 4,532,207, the disclosure of which is herein incorporated by reference]. Sassenfeld and Brewer reported a carboxy-terminal extension of five **arginine** residues fused to a protein of interest (urogastrone). This basic polyarginine extension allowed the purification of the fusion protein on a SP-Sephadex resin. An analogous protein expression and purification system employs a polyhistidine tract or tag at either the amino- or carboxy-terminus of the fusion protein. The fusion protein is purified by chromatography on a Ni.sup.2+ metal affinity resin [Porath, J., Protein Expression and Purification 3:7995 (1992)]. The use of small polypeptides as fusion partner (e.g., the polyarginine or polyhistidine tag) may be insufficient to stabilize a wide variety of foreign proteins in prokaryotes since a fusion protein construct with only ten amino acids from beta-gal was insufficient to stabilize somatostatin [K

[redacted]

following sequences: Ile Glu Gly Arg X; Ile Asp Gly Arg X; and Ala Glu Gly Arg X, where X is any amino acid except proline or arginine [redacted]

amino-terminal residues of the cII protein, a Factor Xa cleavage site and human .beta.-globin was shown to be cleaved by Factor Xa and generate authentic .beta.-globin [Nagai, K. and Thogersen, H. C., Nature 308: 810-812 (1984)].

SUMM In order to cleave some fusion proteins which contain a Factor Xa cleavage site, **denaturation** of the fusion protein is required. It is likely that **denaturation** of the fusion protein permits the protease to gain access to the cleavage site. The need to treat fusion proteins with harsh **denaturants**, such as guanidine hydrochloride or urea, is undesirable. Furthermore, exposing the recombinant protein to harsh **denaturants** may alter the functional activity and/or the antigenicity of the purified protein. In addition, once **denatured**, many proteins do not **renature** (i.e., they become irreversibly **denatured** or unfolded).

SUMM The insertion of a linker or spacer between the Factor Xa site and the protein of interest has been reported to facilitate the cleavage of some fusion proteins. However, the insertion of the linker results in the addition of extra amino acids (i.e., not naturally occurring) at the amino terminus of the protein of interest (Riggs, P., supra at 16.6.13). Another limitation of the Factor Xa-based fusion systems is the fact that Factor Xa has been reported to cleave at **arginine** residues that are not present within in the Factor Xa recognition sequence [Nagai, K. and Thogerson, H. C., supra; Lauritzen, C. et al., Prot. Expr. and Purif. 2:372 (1991)]. Additionally, Factor Xa will not cleave at the recognition site if the site is followed by a proline or **arginine** residue (Riggs, P., supra at 16.6.13).

SUMM The present invention relates to compositions and methods for producing authentic proteins by recombinant means. The invention provides novel fusion proteins and recombinant DNA vectors encoding the same, as well as, methods for the production of authentic proteins from the novel fusion proteins. In one embodiment the invention provides fusion proteins comprising three domains joined together in order from amino-terminus to carboxy-terminus of a first domain comprising a protein of interest, a second domain comprising a hydrophilic spacer, and an affinity domain, each domain comprising amino acid residues. It is not required that each of these domain be contiguous with one another. The invention contemplates fusion proteins wherein additional domains and/or elements (e.g., a penultimate enhancer and/or a CPB terminator) are inserted between the three domains described above. The invention further contemplates a fusion protein wherein the hydrophilic spacer is an **arginine** residue and the hydrophilic spacer and the affinity domain are separated by a domain comprising 1 to 19 amino acid residues wherein these 1 to 20 residues are capable of removal by a means for selective amino acid removal. In a preferred embodiment these 1 to 20 residues are removable by a selective endoprotease cleavage and/or a carboxypeptidase, the latter is preferably selected from the group comprising carboxypeptidase A, carboxypeptidase B and carboxypeptidase Y.

SUMM In particularly preferred embodiment, the susceptible amino acids of the hydrophilic spacer are selected from the group consisting of **arginine** and lysine. In one embodiment, the susceptible amino acids of the hydrophilic spacer have the sequence selected from the group comprising SEQ ID NOS:16-37. The hydrophilic spacers of the novel fusion proteins may comprise an extended hydrophilic spacer. In a preferred embodiment, the extended hydrophilic spacer comprises the

SUMM The invention also provides recombinant DNA vectors having a nucleotide sequence encoding a fusion protein comprising three domains joined

hydrophilic spacer, and affinity domain, each domain comprising amino acid residues. In a preferred embodiment, the recombinant DNA vector encodes a fusion protein wherein the amino acids of the encoded hydrophilic spacer are susceptible to removal by a means for selective amino acid removal, the later preferably being a carboxypeptidase. In another preferred embodiment, the amino acids comprising the encoded hydrophilic spacer are removable using a carboxypeptidase selected from the group comprising carboxypeptidase A, carboxypeptidase B and carboxypeptidase Y. In yet another preferred embodiment, the recombinant vector encodes a fusion protein wherein the susceptible amino acids of the encoded hydrophilic spacer are selected from the group consisting of **arginine** and lysine; particularly preferred encoded hydrophilic spacers comprises sequences selected from the group comprising SEQ ID NOS:16-37. The encoded hydrophilic spacer may comprise an extended hydrophilic spacer; in a preferred embodiment the encoded extended hydrophilic spacer comprises the amino acid sequence of either SEQ ID NOS:18 or 19 in combination with any of SEQ ID NOS:16-37 wherein SEQ ID NOS:18 or 19 are linked via their amino-terminus to the carboxy-terminus of SEQ ID NOS:16-37 and joined via their carboxy-terminus to the affinity domain.

DRWD FIG. 31 depicts the nucleotide and amino acid sequence of human **preproNGF**.

DETD The term "hydrophilic" when used in reference to amino acids refers to those amino acids which have polar and/or charged side chains (i.e., R groups). Hydrophilic amino acids include lysine, **arginine**, histidine, aspartate (i.e., aspartic acid), glutamate (i.e., glutamic acid), glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine.

DETD The term "hydrophilic spacer" refers to combinations of 1 to 5 predominantly hydrophilic amino acids present within the fusion proteins of the present invention, wherein at least one of the amino acid residues is an **arginine** residue. Preferred hydrophilic spacers comprise 3 to 5 hydrophilic amino acids. The term "extended hydrophilic spacer" refers to combinations of 6 to 8 predominantly hydrophilic amino acids. Particularly preferred hydrophilic spacers and/or extended hydrophilic spacers comprise only **arginine** and lysine residues; **arginine** and lysine residues are effectively removed by CPB. The hydrophilic spacers of the present invention contain at least one **arginine** residue; the **arginine** residues provide barriers or termination points for CPA digestions (i.e., CPA cannot remove **arginine** residues). Authentic proteins of interest are generated from the fusion protein by selective removal of non-authentic amino acids from the carboxy-terminus of the fusion protein (after the fusion protein has been cleaved by the desired endoprotease). The **arginine** residue(s) within the hydrophilic spacer acts as a barrier to excessive digestion (i.e., digestion into the protein of interest) of the fusion protein by CPA. When CPA encounters an **arginine** residue it cannot proceed. At that point CPB, which can only remove **arginine** and lysine residues, is used to digest the remaining **arginine** and/or lysine residues of the hydrophilic spacer to generate the authentic protein of interest. As discussed further below, doublets of lysine residues, which are extremely resistant to carboxypeptidase Y (CPD-Y) digestion, may be employed in the hydrophilic spacers. Hydrophilic spacers containing lysine doublets are employed in level 3 linker processing designs which requires the use of CPD-Y to the generation of authentic proteins.

DETD In addition to providing a means for generating authentic proteins by providing residues which are capable of selective removal (e.g., using carboxypeptidases), the hydrophilic and basic nature of **arginine**

[illegible] is formed by the juxtapositioning of an amino acid residue which is slowly released from the endoprotease recognition sequence [illegible]

is also slowly released (e.g., **arginine** and/or lysine residues), the result is an amino acid pair that is processed extremely slowly in the carboxypeptidase reaction (CPD-Y and CPA). In order to increase the speed and efficiency of transition from CPD-Y to CPA to CPB digestion, a preferred amino acid (i.e., a penultimate enhancer) is added at the junction between the hydrophilic spacer and the endoprotease recognition sequence (see FIG. 36 for an example). The residue which functions as the penultimate enhancer will increase the rate at which the amino-terminal residue of the endoprotease site is removed by digestion with carboxypeptidase.

DETD The term "CPB terminator" refers to a single amino acid that prevents the digestion of any authentic protein sequences when removing the amino acid residues comprising the hydrophilic spacer with carboxypeptidase B (CPB). CPB removes only **arginine** and lysine residues. Amino acids which are particularly preferred as CPB terminators are hydrophobic aliphatic residues (e.g., leucine, isoleucine, valine) as these residues are removed quickly by carboxypeptidase A (CPA) and carboxypeptidase Y (CPD-Y). A hydrophobic aliphatic residue at this position will also prevent any cleavage at the authentic molecule linker junction site by furin should the design be used in a mammalian host system and the desired molecule contain a furin recognition motif directly at its carboxy-terminus. When the protein of interest to be expressed in the fusion protein does not contain a furin recognition site or when a non-furin producing host cell is employed, any amino acid that is rapidly released by CPA and that is not released by CPB can be used as a CPB terminator (i.e., phenylalanine, tryptophane, leucine, isoleucine, valine, alanine and methionine). A CPB terminator is employed in the linker design when the protein of interest contains an **arginine** or lysine at its carboxy terminus; the CPB terminator is located on the carboxy-terminal side of the authentic **arginine** or lysine, between the authentic protein of interest and the hydrophilic spacer (see FIG. 34 for an example).

DETD The hydrophilic spacers of the present invention comprise one to five **arginine** and/or lysine residues. Extended hydrophilic spacers comprise six to eight **arginine** and/or lysine residues. The hydrophilic spacers serve several functions. The hydrophilic amino acids which comprise the hydrophilic spacer serve to orient this portion of the fusion protein toward the exterior of the molecule in aqueous solutions; this increases the exposure and accessibility of the nearby endoprotease recognition site. The hydrophilic spacers also allow for the physical separation of the domain comprising the protein of interest from the affinity domain. This separation ensures that the affinity domain is free to interact with the affinity resin as the possibility of steric hinderance from the protein of interest is reduced. In addition, the hydrophilic spacers allow for the physical separation of the endoprotease recognition site from the carboxy-terminal portion of the protein of interest. This separation is advantageous as the carboxy-terminal portion of the protein of interest may limit access of the endoprotease to the endoprotease recognition site if located in close proximity.

DETD 3) Clostropain, which cleaves on the carboxy-terminal side of **arginine** residues, with the preferred sequence being Arg-Tyr.

DETD 4) Trypsin, which cleaves on the carboxy-terminal side of **arginine** and lysine residues.

DETD 6) Kallikrein, which preferentially cleaves on the carboxy-terminal side of **arginine** within the recognition sequence Phe-Arg-Ser-Val (SEQ ID NO:9). When kallikrein is used as the protease for cleavage, the preferred linker sequence is Val-Pro-Phe-Arg-Ser (SEQ ID NO:10). The valine residue present in SEQ ID NO:10 functions as a penultimate enhancer thereby enhancing the removal of the proline residue by CPD-Y

DETD [illegible] which cleaves between the **arginine** and glycine residues with the preferred X' being Leu, Phe, Ile, Val, Ala or Trp [Kuks, P., et al., [illegible]

arginine

residues in the following sequences: Ile-Glu-Gly-Arg-X (SEQ ID NO:4), Ile-Asp-Gly-Arg-X (SEQ ID NO:5), and Ala-Glu-Gly-Arg-X (SEQ ID NO:6), where X is any amino acid except proline or **arginine**.

DETD The Level 1 linker design is employed when the protein of interest is not susceptible to digestion by one of the endoproteases listed in Table 1 and either 1) the naturally occurring carboxy-terminal amino acid of the protein of interest is an **arginine** or a lysine or 2) a spacer comprising basic amino acids is used to link the protein of interest and the affinity purifiable domain. When the protein of interest naturally terminates in an **arginine** or lysine residue, a Level 1 linker can be employed which places an **arginine** or lysine residue next to the carboxy-terminal residue of the protein of interest; in this way a cleavage site for OmpT and/or protease VII is created. Cleavage of such a fusion protein with the OmpT protease or protease VII will generate an authentic protein of interest without the need to further treat the released protein of interest. When the protein of interest is not susceptible to digestion by one of the endoproteases listed in Table 1 but does not contain a carboxy-terminal **arginine** or lysine residue, a Level 1 linker is employed to join the protein of interest to the affinity domain. In this case, sequences encoding the affinity domain are joined to sequences encoding the protein of interest using a linker which encodes basic amino acid residues.

DETD FIG. 1 provides a schematic illustrating Level 1 processing. FIG. 1 shows an exemplary case where the hydrophilic spacer/endoprotease site employed contains a recognition site for a dibasic protease and the affinity domain comprises the hinge and Fc domains of a IgG. In FIG. 1, step 1 shows the fusion protein (as a dimer of two molecules as the IgG sequences are capable of dimerization) bound to the affinity resin (e.g., protein A-Sepharaose). In Level 1 processing, cleavage of the fusion protein generates a released protein of interest which contains either an **arginine** or a lysine residue at the carboxy-terminus (FIG. 1, step 2). Authentic protein of interest is generated from the released protein of interest by removal of the linker-encoded **arginine** or lysine residues (i.e., the residues comprising the hydrophilic spacer) by digestion with carboxypeptidase B.

DETD There are processing advantages to using the enzymes listed in Table 1 above. These enzymes recognize the amino acids **arginine** and/or lysine without the requirement for specific amino acids in positions located toward the amino-terminus of the substrate. As discussed below, generation of authentic amino acid products is achieved by incubating the cleaved fusion protein with immobilized carboxypeptidase B, thus removing the amino acids comprising the hydrophilic spacer. Dibasic recognition proteases (i.e., yeast Kex2, OmpT and protease VII) are preferred over trypsin due their increased specificity. The OmpT protease is a dibasic recognition protease which is readily isolated from the outer membrane of any E. coli K strain which expresses the protease, such as LE 392 (Stratagene), by incubating whole cells with 30 mM n-octylglucoside [Grodberg J. and Dunn J. J., J. Bacteriol. 170:1245 (1988)].

DETD Level 2 spacer/endoprotease site (i.e., linker) designs are used in combination with endoproteases that leave a portion of their recognition sequence behind after proteolytic cleavage. This remnant, because of its amino acid sequence, can be removed by sequential treatment with carboxypeptidase A (CPA) and carboxypeptidase B (CPB). CPB removes carboxy-terminal **arginine** or lysine residues only. CPA can rapidly digest or remove carboxy-terminal tyrosine, phenylalanine, tryptophan, leucine, isoleucine, methionine, threonine, glutamine, histidine, alanine and valine residues. CPA removes carboxy-terminal asparagine, serine and lysine slowly; glycine, aspartic acid, glutamic

[illegible] CPB or CPA can remove. Combination of amino acids which are released very slowly or not at all released amino acids proline or **arginine** [illegible]

addition of the leucine residue into the enterokinase linker allows CPA to proceed smoothly to the **arginine** residue by avoiding the extremely slow step of Arg-Asp (the CPA digestion is conducted at 37.degree. C.).

DETD FIG. 2 provides a schematic which represent the generation of authentic protein using the Level 2 spacer design. In FIG. 2, basic amino acids which can be removed by CPB are represented by the circles and amino acids which can be removed by CPA are represented by the squares. Level 2 processing is illustrated using a hydrophilic spacer which comprises the sequence Arg-Arg-Lys (SEQ ID NO:16); the spacer is followed by a leucine residue which functions as a penultimate enhancer; the penultimate enhancer is followed by the recognition site for the endoprotease enterokinase [Asp-Asp-Asp-Asp-Lys (SEQ ID NO:13)]. Step 1 of FIG. 2 shows the released protein of interest generated by digestion of the fusion protein with enterokinase (enterokinase cleaves on the carboxy-terminal side of the lysine residue present in the enterokinase recognition site); the released protein is then treated with CPB to remove the terminal lysine residue. Step 2 of FIG. 2 shows the released protein of interest following treatment with CPB and indicates that the released protein of interest is now to be treated with CPA to remove the asparagine and leucine residues. In all Level 2 and 3 designs, the preferred hydrophilic spacer has a lysine residue at its carboxy terminal position to allow efficient transition from CPA digestion to CPB digestion. Carboxy terminal lysine residues can be removed with CPA and/or CPB. The lysine residues allow CPA to proceed completely through the remaining exoprotease recognition sequence or penultimate enhancer without any inhibition. An **arginine** residue in the same position would slow the reaction and therefore is not preferred. Complete efficient removal of the remaining exoprotease recognition sequence or penultimate enhancer ensures that the hydrophilic spacer will be available for CPB digestion. Following treatment with CPA, the released protein of interest is treated again with CPB to remove any remaining lysine residues and the **arginine** residues (step 3) to generate the authentic protein of interest (step 4). As discussed in greater detail below, removal of the amino acids which comprise the endoprotease site and the hydrophilic spacer can be achieved using immobilized forms of the carboxypeptidases. The use of immobilized enzymes is advantageous as this obviates the need to remove the carboxypeptidases from the final preparation of the authentic protein and allows the sequential digestion of the released protein of interest with the carboxypeptidases.

DETD Level 2 designs are used when the protein of interest would be susceptible to the cleavage protocol described above for the Level 1 design. Level 1 linkers comprise hydrophilic spacer sequences that do not require additional endoprotease sequences because the endoproteases used in the Level 1 design recognize and cleave the hydrophilic spacers. Level 2 linkers encode protease recognition sites for proteases that leave amino acids on the carboxy-terminus of the protein of interest which cannot be removed by digestion with CPB. Level 2 denotes that additional in vitro processing steps are needed to generate authentic protein molecules, specifically CPA digestion(s) is required. Due to the specificities of the carboxypeptidases and the digestion conditions utilized in conjunction with the Level 2 and 3 linkers it is not possible to generate authentic proteins that have carboxy terminal lysine residues using carboxypeptidases to digest non-authentic amino acid residues from the protein of interest. All of the currently characterized carboxypeptidases can remove lysine residues under the conditions described herein. However, the Level 1 linker design that inserts a single **arginine** residue after the naturally occurring lysine residue to create an OmpT/proteases III

[illegible] Lys Lys Lys (SEQ ID NO:18). This hydrophilic spacer is placed following the natural **arginine**, and allows the hydrophilic spacer to be [illegible]

arginine residues and requires alternating CPA and CPB digestions to generate authentic protein with a carboxyterminal **arginine**. A leucine residue is placed between the natural **arginine** and the **arginine** represented in the hydrophilic spacer Arg-Lys-Lys SEQ ID 16) in order to act as a termination point for the CPB digestion. CPB is used as described to remove the hydrophilic spacer, stopping at the inserted leucine residue. A final CPA digestion is used to remove the leucine residue and generate an authentic protein.

DETD Step 1 of FIG. 3 shows the released protein of interest generated by digestion of the fusion protein with renin (renin cleaves on the carboxy-terminal side of the first leucine residue present in the renin recognition site); this protein is then treated with CPA to remove the leucine, histidine and phenylalanine residues which remain after digestion of the fusion protein with renin. This first CPA digestion is allowed to go to completion as the proline residue will halt digestion by CPA. The CPA-treated released protein is then treated with CPD-Y to remove the terminal proline residue (Step 2 of FIG. 3); the use of the leucine residue as a penultimate enhancer allows the efficient digestion of proline by CPD-Y. Following treatment with CPD-Y, the protein of interest is treated with CPA to remove the leucine residue. The lysine and **arginine** residues of the hydrophilic spacer are then removed by digestion with CPB (Step 4) to generate the authentic protein of interest (Step 5).

DETD The following are preferred forms of hydrophilic spacer sequences: Arg-Arg-Lys (SEQ ID NO:16); Arg-Lys-Lys (SEQ ID NO:17); Lys-Arg-Lys (SEQ ID NO:18); Lys-Lys-Lys (SEQ ID NO:19); Arg-Arg-Arg-Lys (SEQ ID NO:20); Arg-Arg-Lys-Lys (SEQ ID NO:21); Lys-Arg-Arg-Lys (SEQ ID NO:22); Arg-Lys-Arg-Lys (SEQ ID NO:23); Arg-Lys-Lys-Lys (SEQ ID NO:24); Lys-Arg-Lys-Lys (SEQ ID NO:25); Lys-Lys-Arg-Lys (SEQ ID NO:26); Arg-Arg-Arg-Arg-Lys (SEQ ID NO:27); Arg-Arg-Arg-Lys-Lys (SEQ ID NO:28); Arg-Arg-Lys-Arg-Lys (SEQ ID NO:29); Arg-Lys-Arg-Arg-Lys (SEQ ID NO:30); Lys-Arg-Arg-Arg-Lys (SEQ ID NO:31); Arg-Arg-Lys-Lys-Lys (SEQ ID NO:32); Arg-Lys-Arg-Lys-Lys (SEQ ID NO:33); Arg-Lys-Lys-Arg-Lys (SEQ ID NO:34); Lys-Arg-Arg-Lys-Lys (SEQ ID NO:35); Lys-Arg-Lys-Arg-Lys (SEQ ID NO:36); Lys-Arg-Arg-Lys-Lys (SEQ ID NO:37); and Arg-Lys-Lys-Lys-Lys (SEQ ID NO:38). These preferred hydrophilic spacers can be used in Level 1, 2 or 3 linker designs; these spacers can be used when the fusion protein is to be expressed in non-endocrine mammalian cell lines. Fusion proteins comprising proteins of interest which end in an **arginine** or lysine residue require the insertion of a leucine residue between the carboxy-terminal **arginine** or lysine of the protein of interest and the hydrophilic spacer (as described above for Level 2 designs).

DETD The above listed sequences represent preferred spacer sequences which should be adequate for separating the desired endoprotease site from the carboxy-terminus of the protein of interest. The invention also contemplates the insertion of hydrophilic triplets such as Lys-Lys-Lys (SEQ ID NO:19) and Lys-Arg-Lys (SEQ ID NO:18) to the amino-terminal end of any of the above-listed spacers to generate extended hydrophilic spacers. These longer (i.e., extended) spacers are employed when the carboxy-terminus of the protein of interest is sufficiently buried within the hydrophobic interior of the protein so as to structurally inhibit the removal of any remaining endoprotease recognition sequences and/or the penultimate enhancer by CPA digestion. Traditional approaches to dealing with the cleavage of fusion proteins having a buried carboxy-terminus of the protein of interest employ the use of **denaturant** during the digestion of the fusion protein. This approach is not appropriate when CPA is to be employed as CPA loses most of its activity under **denaturing** conditions. The use of the "extended hydrophilic spacers" is appropriate when the protein of

digestion of these sequences with CPA under **denaturing** conditions. The extended hydrophilic spacer can be removed by digestion with CPB under **denaturing** conditions (e.g., in the presence of

preferred method for rem[redacted]l of the affinity domain until[redacted] carboxypeptidases which cannot remove lysine residues become available.

DETD The production of recombinant proteins often involves the use of protease inhibitors to prevent the degradation of the recombinant protein (e.g., fusion protein) before it can be isolated in a purified form. Numerous protease inhibitors are known to the art and include, but are not limited to leupeptin, pepstatin A, antipain, aprotinin, PEFABLOC (Pentapharm Ltd., Basel, Switzerland), chymostatin, trypsin inhibitor from soybean, FBS-d-PI, phenylmethylsulfonyl fluoride (PMSF) and (4-amidinophenyl) methane sulfonyl fluoride (APMSF). Due to the design of the hydrophilic spacers of the present invention, it is required that steps are taken to inhibit trypsin and other serine proteases that recognize **arginine** and/or lysine residues to prevent the cleavage of the fusion proteins. In selecting a cell line to be used as a host cell for the production of fusion proteins, the cell line is screen for the ability to produce and/or secrete proteases which can cleave the hydrophilic spacers of the invention. In addition, medium supplements should also be monitored for the presence of these proteases. Cell lines (and culture supernatant from cell lines) and medium supplements can be monitored using commercially available synthetic peptide substrates. Four particularly useful synthetic substrates are N-benzoyl-Val-Lys-Lys-Arg-4-methoxy-B-napthyamide, N-t-Boc-Glu-Lys-Lys-7-amido-4-methycoumarin, N-t-Boc-Gly-Arg-Arg-7-amido-4-methylcoumarin and N-t-Boc-Gly-Lys-Arg-7-amido-4-methylcoumarin [Mizuno et al., Biochem. Biophys. Res. Commun. 144:807 (1987)]; all of these substrates are available from Sigma. Cell lines and medium supplements which express the least amount of protease activity on these type of substrates (i.e., substrates containing **arginine** and/or lysine residues) are preferred.

DETD Insect cells which lack protease activity have not been reported. Accordingly, when fusion proteins are to be expressed in insect cells [e.g., Sf9, Sf21 and MG1 cells (Stratagene)] the following hydrophilic spacers are used: Arg-Lys-Lys (SEQ ID NO:17), Arg-Lys-Lys-Lys (SEQ ID NO:24) and Arg-Lys-Lys-Lys-Lys (SEQ ID NO:38). If an extended hydrophilic spacer is to be employed for the expression of fusion proteins in insect cells, the lysine triplet (SEQ ID NO:19) can be added to the carboxy-terminal end of the above 3 spacers. The ability of the Sf9 insect cell line to at least partially process **proNGF** into authentic, active NGF by cleavage of the naturally occurring proprocessing site Arg-Ser-Lys-Arg (SEQ ID NO:39) (U.S. Pat. No. 5,272,063, the disclosure of which is herein incorporated by reference) limits the use of hydrophilic spacers to those containing Arg-Lys and Lys-Lys amino acid combinations and those lacking Arg-Arg and Lys-Arg combinations.

DETD The presence of a dibasic recognition site alone is not sufficient to allow proteolytic cleavage as many hormones and growth factors have internal dibasic sites (i.e., sites located within the sequences encoding the mature form of the protein) that are not cleaved during secretion. A study of sequences encoding prosomatostatin derived from several species suggests that the general exposure (i.e., location on the exterior of the molecule) and conformation of the dibasic site may influence whether a particular site is susceptible to cleavage [Warren, Cell 39:547 (1984)]. The enzymes responsible for dibasic cleavage in the constitutive secretion pathway (i.e., non-regulated secretion) have been characterized; these enzymes are termed furin or PACE. Furin and PACE require an **arginine** at the P4 site for cleavage [Hatsuzawa et al., J. Biol. Chem. 267:16094 (1992)]. The specificities of furin and PC1/PC3 enzymes from the endocrine system have been compared [Nakayama, J. Biol. Chem. 267:16335 (1992)] and found to be similar [the recognition sequence for furin is Arg-X-Lys/Arg-Arg (SEQ ID NOS:14 and

[illegible] specificity, [illegible] amino acids from the carboxy terminus of recombinant fusion proteins following cleavage with endoproteases. CPA releases different amino acids at different rates (Ambler, supra). The [illegible] for the [illegible] by CPA [illegible]

glutamine, histidine, alanine, valine and homoserine. The following amino acids are releases slowly by CPA: asparagine, serine, lysine (the rate of lysine release may be modified by changing the pH of the digestion) and MetSO.sub.2. The following amino acids are released very slowly by CPA: glycine, aspartic acid, glutamic acid, CySO.sub.3 H and s-carboxymethylcysteine. The following amino acids are not released by CPA: proline, hydroxyproline and **arginine**. The presence of an amino acid which is either very slowly released or not released in the penultimate position will generally decrease the rate of release of the carboxy-terminal residue by CPA. CPB has a much more narrow specificity as compared to CPA; CPB removes only **arginine** and lysine residues rapidly (Ambler, supra).

DETD CPA and CPB have defined limitations as to their removal of carboxy-terminal amino acids and are used to digest remaining linker sequence to completion, therefore traditional immobilization media such as activated CNBr agarose beads can be used. Immobilized CPA digestions can be incubated to completion because the hydrophilic spacers protect the protein of interest by encoding an **arginine** residue which CPA cannot remove. (All hydrophilic spacers contain at least one **arginine**; the lysine triplet used to generate an extended hydrophilic spacer is used in combination with spacers which contain an **arginine** or alternatively may be used as the spacer when the protein of interest terminates with an **arginine** residue). Alternate immobilization media is needed to control the hydrolysis of the carboxy-terminal amino acids when CPD Y is used as the exoprotease, because CPD-Y does not have the specific substrate limitations of CPA and CPB. CPD-Y attached to traditional immobilization media (e.g., agarose) produces a wide variety of digestion products. This heterogenous population of digested products is useful when attempting to determine the organization of amino acids at the carboxy terminus (i.e., for determination of protein sequences). Extensive proteolytic digestion is likely to occur as result of the peptide entering into diffusion zones were the enzyme concentration is high and the rate of diffusion is slow. The desired effect when performing CPD-Y digestions is a uniform, but limited, removal of a specific amino acid (proline) from a large homogenous population of molecules. This can only be accomplished by limiting the time that a high uniform concentration of the CPD-Y enzyme is allowed to interact with limiting concentrations (i.e., below the K.sub.m) of substrate.

DETD Carboxypeptidase A can release a wide variety of amino acids from the carboxy terminus at varying rates, except proline and **arginine** (Ambler R. P., supra). The strategy of alternating between carboxypeptidase A and B is used when the cleavage sequence does not contain any prolines. The enterokinase recognition sequence used in Level 2 designs is an example of this strategy. The sequence Arg-Arg-Lys-Leu-Asp-Asp-Asp-Asp-Lys (SEQ ID NO:41) remains after cleavage of the fusion protein (see FIG. 2). The lysine residue can be removed by digestion with CPA or CPB at pH 8.0 at 25.degree. C. The release of the lysine, asparagine and leucine residues by CPA is very slow at room temperature, but the reaction rate can be increased by raising the temperature to 37.degree. C. and lowering the pH to less than 6.2 (Ambler R. P., supra). The reaction can be allowed to go to completion (stopping at the **arginine** residues) as long as suitable protease inhibitors are present (i.e., disopropylflourophosphate). Authentic protein is generated by removing the remaining **arginine** residues with carboxypeptidase B.

DETD In circumstances where carboxypeptidase A cannot remove the remaining amino acids from the protease recognition sequence, alternate digestion protocols are used. Since the sequence of amino acids to be removed from the protein of interest is known, the enzymes used are chosen based on

arginine

to remove the proline residue. The reaction is slow, but having a valine residue in the penultimate position enhances the binding and cleavage rate. The lysine triplet not only provides a hydrophilic

preferred amino acid to [redacted]ve in the penultimate position [redacted]nus, the lysine pair is a formida[redacted]e obstacle for CPD-Y digestion. Multiple passes (about 3 or 4) of the cleaved protein through an immobilized carboxypeptidase Y medium at a rate suitable to remove the carboxy-terminal proline insures that the digestion will go to completion (i.e., approximately 100% past proline and approximately 0% past **arginine**). Immobilized CPA is used to remove any remaining leucine, valine and lysine residues and a final digestion with CPB is used to generate the authentic protein.

DETD Cleavage with the site-specific endoprotease may leave extra amino acids on the carboxy-terminal end of the protein of interest (i.e., for Level 2 and 3 designs). These amino acids remain as a result of the amino acids present on the amino-terminal side of the cleavage site for the site-specific endoprotease as well as those within the hydrophilic spacer. These undesirable (i.e., non-authentic) amino acids are removed by digestion with carboxypeptidases. Carboxypeptidases cleave carboxy-terminal amino acids. Carboxypeptidase A cleaves carboxy-terminal amino acids other than **arginine** or proline. Carboxypeptidase B cleaves only carboxy-terminal **arginine** or lysine residues. For example, if the fusion protein is cleaved at the following thrombin site: Leu-Val-Pro-Arg-Gly-Thr (SEQ ID NO:43) located within the following sequence: Protein of interest-Arg-Arg-Lys-Lys-Lys-Leu-Val-Pro-Arg-Gly-Thr-IgG hinge/Fc, then following cleavage with thrombin, the protein of interest will have the following extra carboxy-terminal amino acids: Protein of interest-Arg-Arg-Lys-Lys-Lys-Leu-Val-Pro-Arg. Treatment with immobilized carboxypeptidase B will remove the first **arginine** residue. Digestion with carboxypeptidase Y at pH 5.75 will remove the proline residue and most of the valine and leucine residues. Digestion with carboxypeptidase A at pH 6.0 will remove the remaining valine and leucine residues; the enzyme will slow down at the lysine residues. Digestion with carboxypeptidase B will remove any remaining lysine residues and the **arginine** tail yielding an authentic carboxy-terminus of the protein of interest. Alternating carboxypeptidase digestions can be use to generate an authentic protein of interest when the linker utilized contains **arginine** and/or lysine residues following the carboxy-terminus of the protein of interest.

DETD When the natural carboxy-terminus of the protein of interest comprises an **arginine** residue, the linker utilized will contain a leucine, valine or isoleucine residue between the naturally occurring **arginine** on the protein of interest and the **arginine** /lysine residues in the spacer. These residues (Leu, Val, Ile) are preferred when expression of the fusion protein is achieved in a mammalian cell line in order to prevent the possibility of undesirable cleavage of the fusion protein by furin after the **arginine** located at the carboxy-terminus of the protein of interest. During processing of the released protein of interest, carboxypeptidase B will proceed through the hydrophilic spacer residues until it reaches the leucine or tyrosine residue (referred to as a CPB terminator). Carboxypeptidase A is then used to efficiently remove the leucine, valine or isoleucine residue while leaving the naturally occurring **arginine** residue intact as the carboxy-terminal residue of the protein of interest.

DETD The junction region (i.e., the region which joins the protein of interest with the affinity domain) present in pMA2-TH-IgG is shown in FIG. 7. The first 5 amino acid residues shown comprise the carboxy-terminal end of the MBP (the phenylalanine is encoded by the conversion linker as described above). The hydrophilic spacer (Arg-Arg) and thrombin recognition site are boxed and labeled; the cleavage site for thrombin is indicated by the arrow placed between the Arg and Gly

arginine

the hinge region of IgG. The pairs of **arginine** residues surrounding the thrombin site in the linker were designed to allow for

DETD After cell extracts were prepared from induced bacterial cells (as described above), the resulting supernatants were prepared for chromatography by passage through a 0.44 micron filtration cartridge which included a prefilter matrix to prevent clogging [Uniflow Plus, (Schleicher and Schuell)]. The supernatant was the then brought to 450 mM NaCl by adding an appropriate volume of 5 M NaCl. The sample was applied to a 2.0 ml protein A column which had been pre-equilibrated with 5 volumes of binding buffer (50 mM Tris pH 8.0, 450 mM NaCl). The sample was applied at a flow rate of approximately 0.5 ml/min using only gravity. The flow-through was collected and reapplied to the column. The column was washed with 10 volumes of binding buffer and the fusion protein was eluted by the addition of 5 column volumes of elution buffer (0.04 M Citrate buffer, pH 2.8). Fractions (1 ml) were collected into microcentrifuge tubes containing 100 .mu.l of neutralizing buffer (1 M Tris-HCl, pH 9.0) and protein levels were monitored using a micro protein assay kit based on brilliant blue G (Coomassie blue) interaction with protein to produce a blue colored complex (Sigma). Fractions containing eluted protein were pooled and run on an 4-15% precast SDS-PAGE mini gradient gel (Schleicher & Schuell) to determine purity. Samples were boiled for two minutes after adding an equal volume of 2.times. loading buffer (0.5 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 0.01% bromphenol blue). Visual inspection of the PAGE gel after staining with Coomassie brilliant blue dye showed that the fusion protein was isolated in both monomeric and dimeric forms and was greater than 95% pure (gels run under non **denaturing** conditions were used to estimate the percentage of protein present as a dimer). These results demonstrate that both monomeric and dimeric IgG hinge/Fc regions can bind to protein A. Furthermore, the results show that affinity purification of the fusion protein from total cellular extracts is specific for the MBP/IgG fusion protein.

DETD Solutions to this problem involve the **denaturation** of the fusion molecule with guanadinium HCl or 8 M urea before enzymatic cleavage (Riggs, supra). However, the use of harsh **denaturants** can significantly decrease or eliminate the functional activity of the desired protein. Alternatively, other proteases have been used that more efficiently cleave fusion molecules as the result of the cleavage site being towards the middle of a recognition sequence rather than following a recognition sequence (for example, thrombin, renin, Igase). However, these proteases do not generate authentic proteins as following endoprotease digestion amino acids contributed by the endoprotease recognition site remain on the protein of interest.

DETD In contrast, the hydrophilic spacers of the present invention physically separate the natural conformation of a desired molecules carboxy-terminus from the designed proteolytic site and provide enhanced solubility because of their hydrophilic nature. The hydrophilic spacer permits the removal of any residual proteolytic recognition sequence that remains at the carboxy-terminus of the authentic protein after the specific cleavage of designed fusion protein. The **arginine** residue(s) present in the hydrophilic spacer provide a barrier to prevent the removal of residues from the carboxy-terminus of the authentic protein of interest by CPA (Ambler, supra) and allows for the removal of any amino acids derived from the endoprotease recognition site which remain on the carboxy-terminus of the protein of interest following endoprotease digestion of the fusion protein.

DETD Total cellular RNA was isolated from the ARH-77 cell pellet using a Total RNA Isolation Kit (Clonetech) according to the manufacturer's instructions. Briefly, 10 ml of **denaturing** solution (6 M guanidinum-HCl) was added to the pooled, washed cells (10 ml) and incubated for 10 minutes at room temperature. The following reagents were added in the stated order with gentle mixing: 1.0 ml 2 M NaOAc pH

DETD The tube was stored at -20.degree. C. overnight. The RNA was then pelleted by centrifugation at 8000.times.g in a SS34 rotor at 4.degree.

denaturing

(Fisher). The RNA was precipitated by the addition of 0.6 [redacted] of isopropanol and incubation of the tube at -20.degree. C. for 1 hour. RNA was pelleted gently in a Eppendorf microcentrifuge at 5,000.times.g for 10 min at 4.degree. C., resuspended in 75% ethanol and stored at -20.degree. C. until needed.

DETD As shown in FIG. 11, these three oligonucleotide primers introduce different amino acids at the 5' end of the hinge region. The IG5NGO oligonucleotide (SEQ ID NO:51) contains the recognition site for NgoMI and introduces two **arginine** residues immediately upstream of the histidine residue located at amino acid position 225 in the human IgG1 molecule. The IG5ARS oligonucleotide (SEQ ID NO:52) contains the recognition site for SalI and introduces two **arginine** residues at the 5' end of the hinge region (immediately upstream of the threonine residue located at amino acid position 226 in the human IgG 1 hinge region). The IG5KPN oligonucleotide (SEQ ID NO:53) contains a KpnI site and introduces a glycine residue in the hinge region (immediately upstream of the threonine residue located at amino acid position 224 in the human IgG hinge region).

DETD The resulting 0.7 kb PCR products contain three variations of the hinge domain. They are designed to allow the naturally occurring proteolytic cleavage site of the hinge maximum exposure (see FIG. 10). The SalI-IgG product was designed to be very hydrophilic; this product is generated using primers comprising SEQ ID NOS:52 and 54. Two **arginine** residues encoded by the 5' primer (SEQ ID NO:52) were used to replace the naturally occurring Thr(224) and His(225) to make the region more hydrophilic. In the KpnI-IgG fragment a glycine residue encoded by the 5' primer (SEQ ID NO:53) replaces the naturally occurring Lys(223) amino acid to allow for maximum rotation of the protein of interest and attached endocleavage site, Thr(224) and His(225) were not disturbed. The KpnI-IgG fragment is generated using primers comprising SEQ ID NOS:53 and 54.

DETD In the NgoI-IgG fragment, the threonine (at position 224) was replaced with an **arginine** residue to make the hinge region more hydrophilic. A glycine codon (GGG) can be created by using a cloning linker that terminates with GG and has an NgoI compatible 5' overhang to provide additional flexibility.

DETD The 5' linker used to join the IgG fragment to the vector and provide the spacer and endoprotease site may be designed such that additional **arginine**, lysine and tyrosine residues may be placed upstream of the endoprotease site. Insertion of the IgG fragment into the XbaI site in the above described manner allows the remaining sites in the multiple cloning site to be utilized for insertion of sequences encoding the gene of interest. The above-described linker contains a NruI site (TCGCGA) which produces a blunt end upon digestion. The resulting blunt end has CGA as its first three nucleotides which encodes the first **arginine** residue of the hydrophilic spacer. The sequence following this CGA can be varied to generate the desired hydrophilic linker and endoprotease site.

DETD As shown in FIG. 21, pTVBac-kIg retains most of the cloning sites present in the multiple cloning site of the original vector; these sites are available for insertion of the 5' end of the sequences encoding the protein of interest. The translation initiation codon (ATG) must be provided by the sequences encoding the protein of interest. NruI digestion of pTVBac-kIg provides a blunt end for the ligation of the 3' end of the inserted gene while preserving the first **arginine** residue of the hydrophilic spacer. Several variations of hydrophilic spacers and endoprotease sites can be engineered using the approach described above to create specific vectors for the production of fusion proteins which can be isolated using IgG affinity chromatography (i.e., use of Protein A and/or G resins).

[redacted] large surface area of the pores when CPB is immobilized to Sepharose 4F Pharmacia. When a released protein of interest containing an exposed [redacted]

and are acted upon by the immobilized enzyme. Because the diffusion process is slow compared to the enzymatic reaction, the probability that the multiple **arginine** and/or lysine residues of the spacer will be removed while the protein of interest is in the pore is high. This is advantageous when CPA or CPB digestions are to be performed as spacer designs which require treatment with these enzymes require that the reaction go to completion in order to generate authentic protein of interest.

DETD Both NGF and BDNF are synthesized as larger precursor forms (termed **preproNGF** and preproBDNF) which are then processed by proteolytic cleavages, to produce the mature neurotrophic factor. These prepro regions are located at the amino terminus of the precursor molecule and are needed for proper folding and secretion of these proteins. The mature forms of NGF and BDNF have **arginine** residues at their carboxy termini which requires that a leucine residue be inserted between the naturally occurring **arginine** and the hydrophilic spacer. This leucine residue is called a CPB terminator because it prevents CPB from removing authentic amino acids from the natural protein; the CPB terminator can be removed with CPA to generate authentic molecules.

DETD The precursor **preproNGF** molecule is also proteolytically modified at its carboxy terminus to generate the mature **arginine** -terminating NGF molecule. The human gene sequence for the carboxy terminus of the precursor NGF molecule is shown below to code for an extra **arginine** and alanine residues. These two amino acids are removed to generate mature NGF by the dibasic proteolytic activity of the gamma NGF subunit.

DETD Both NGF and BDNF require proteolytic processing and formation of the correct intramolecular disulfide bonds to produce mature fully-biologically-active or mature forms of these proteins. Previous attempts to produce these molecules in bacterial hosts required the expression of truncated mature NGF sequences in bacteria (i.e., sequences which lack the pro regions) and further required inefficient in vitro refolding steps to generate active molecules [See, U.S. Pat. No 5,235,043 and European Patent Application No. 336,324]. The use of eucaryotic cells such as mammalian cells permits the proper proteolytic processing of NGF molecules encoded by the pre-proprotein forms of the gene; however, the expression of the full length **preproNGF** protein in mammalian systems produces low yields of active secreted mature NGF and the use of mammalian cells for the production of proteins is costly [Edwards, et.al., Mol. Cell. Biol. 8:2456 (1988)]. Therefore, it is desirable to produce members of the NGF/BDNF family of proteins in inexpensive host cells such as bacteria. The following example provides methods for the production of human NGF in bacterial host cells without the need to use inefficient in vitro refolding procedures to generate biologically-active (i.e., correctly processed and folded) proteins.

DETD FIGS. 31 and 32 provide the nucleic acid and amino acid sequences of human **preproNGF** and preproBDNF, respectively. The nucleic acid sequence and amino acid sequence of **preproNGF** are listed in SEQ ID NOS:74 and 75, respectively. The nucleic acid sequence and amino acid sequence of preproBDNF are listed in SEQ ID NOS:76 and 77, respectively. The sequence of the mature form of NGF and BDNF is indicated by the use of the large box which encloses the nucleic and amino acid sequences in each figure. In FIGS. 31 and 32, underlining is used to indicated sequences which correspond to sequences present in oligonucleotide primers can be used to generate a DNA sequence encoding the **preproNGF** and preproBDNF, respectively. In FIGS. 31 and 32, amino acids present in the mature form of NGF and BDNF are labeled with positive numbers; negative numbers indicate amino acid residues which are removed during proteolytic processing to generate the mature

proteins contain hydrophilic **arginine** residues at their carboxy termini making it necessary to adjust the composition of the hydrophilic spacer designs when expressing these proteins as fusions with fusion partners that efficiently [illegible] the hinge and [illegible]

to generate authentic carboxy termini and can prevent aberrant cleavages by endogenous proteases present in the production host (i.e., furin in mammalian cells).

DETD The placement of a leucine residue following the carboxy-terminal **arginine** residues present in the NGF and BDNF proteins prevents CPB from removing the natural **arginine**. This hydrophobic aliphatic residue (Leu) would also prevent any processing by furin if the carboxy-terminus contained such a recognition motif (Arg-X-Arg/Lys-Arg SEQ ID NOS:14 & 15). The carboxy-terminal 11 amino acids of the human NGF and BDNF proteins are shown below using the one letter symbol for the amino acids. Sequences shown in bold type are residues encoded by the hydrophilic linker which encodes the hydrophilic spacer which joins the protein of interest to the affinity domain (the KpnI/NheI IgG fragment) via sequences encoding an endoprotease site.

DETD The sequence Leu-Lys-Arg-Arg (SEQ ID NO:78) represents the preferred linker when 1) the desired protein has an **arginine** amino acid at its natural carboxy terminus, 2) the mature protein is not susceptible to the dibasic cleavage protocol and 3) the desired host is a strain E. coli deficient in proteolysis (i.e., AG1). The hydrophilic spacer (Lys-Arg-Arg; SEQ ID NO:79) within the preferred linker contains two endoprotease sites susceptible to the Kex2 protease. The sequence Leu-Lys-Lys-Lys (SEQ ID NO:80) represents a preferred linker when the protein of interest ends with **arginine** and is going to be expressed in host that expresses furin or furin-like proteases. This linker contains a leucine residue and the hydrophilic spacer Lys-Lys-Lys (SEQ ID NO:19), both of which can be removed by CPA digestion. Authentic forms of mature NGF and BDNF are generated from the above-described fusion proteins by digestion with an endoprotease followed by digestion with one or more carboxypeptidases. The leucine residue (L) following the carboxy-terminal **arginine** (R) is removed from the protein of interest with a final carboxypeptidase A digestion (described in detail below).

DETD DNA sequences encoding the **proNGF** protein (i.e., amino acid residues -104 to 108, see FIG. 31) is inserted into the pTVkIgG-1 expression vector (described in Example 4a) to produce a fusion protein containing a carboxy-terminal IgG fragment that is secreted into the periplasmic space where proper folding and disulfide bond formation may occur. The resulting expression vector is termed pTV-TH-NGF.

DETD The fusion protein encoded by pTV-TH-NGF comprises (from amino to carboxy-terminus) the pho signal sequence, the **proNGF** protein sequence, a CPB terminator (Leu), a hydrophilic spacer comprising the sequence Lys-Arg-Arg (SEQ ID NO:79), and the hinge and Fc domains of human IgG1. The hydrophilic spacer in this situation is also the designed endoprotease site(s) for the Kex2 protease. The resulting fusion protein is directed to the periplasmic space due to the presence of the pho signal sequence; the pho signal sequence is cleaved from the fusion protein during transport to the periplasm. Transport to the periplasmic space allows for the proper folding and disulfide bond formation within NGF sequences (without the need to use in vitro refolding procedures). The fusion protein is recovered from the periplasmic space and affinity purified on a Protein A resin. NGF-Leu-Lys-Arg is released from the Protein A resign and separated from its pro region by recirculating a commercially available Lys-Arg and Arg-Arg specific protease (i.e., the Kex2 dibasic protease from yeast which is available from Mo Bi Tec, Gottingen, Germany) through the Protein A resin. The pro region of the **proNGF** protein sequences (i.e., amino acid residues -104 to -1, see FIG. 31) contains a furin processing site Arg-Ser-Lys-Arg (SEQ ID NO:39) that will be correctly cleaved at the carboxy terminal side of **arginine** by the Kex2 protease. Heterologous sequences present on the NGF

proNGF

protein is isolated using the PCR. A mouse prostate cDNA library (Clontech) is used as the template in the PCR. Oligonucleotide primers which bracket the sequences encoding the **proNGF** protein are

preproNGF protein

which are used to amplify the **proNGF** gene are underlined in FIG. 31.

DETD Alternatively, RNA from a human source of Schwann cells known to contain the NGF mRNA can be used to generate first strand cDNA as described in Example 3; this single stranded cDNA preparation is then used as the template in a PCR to permit isolation of sequences encoding the **proNGF** protein.

DETD Nucleic acid sequences (e.g., cDNA) encoding the proprotein form of NGF are isolated using the PCR as follows (it is noted that it is not necessary to isolate the DNA prior to use in the PCR as described below; a phage lysate may also be employed). A five microliter aliquot of HindIII-digested phage library DNA or first strand cDNA (prepared as described in Example 3) are amplified in a final reaction volume of 100 .mu.l containing 10 .mu.l 10.times. Pfu amplification buffer (Stratagene), 0.5 .mu.M each primer [Ngf1 (SEQ ID NO:75) and Ngf2 (SEQ ID NO:76), 200 .mu.M of each of the four dNTPs and 1 unit of Pfu polymerase (Stratagene). The reaction mixture is heated to 94.degree. C. in a thermal cycler (Perkin-Elmer) for 4 minutes to completely **denature** the target DNA and subsequently cycled 30 times (94.degree. C. for 90 seconds, 50.degree. C. for 90 seconds and 72.degree. C. for 2.5 minutes). Two microliters of the PCR products are run on a 2% agarose gel to analyze the amplified product. The PCR products may be digested with restriction enzymes; restriction digestion of the desired **proNGF** PCR products (which are approximately 660 bp in length) with EcoRI will produce two approximately 330 bp fragments that will appear as a doublet on the agarose gel.

DETD Amplified **proNGF** DNA fragments are purified by electrophoresing the amplified reaction products on a 1.5% LMA TAE agarose gel. The approximately 660 bp DNA fragment is cut from the gel and digested with Gelase following the manufacturers protocol (Epicentre Technologies). The 5' end of the NgfI oligonucleotide (SEQ ID NO:81) primes the NGF gene at the beginning of the pro region (Glu at position -104; see FIG. 31) and because Pfu polymerase has 3'-5' exonuclease activity, it produces a blunt end product that is ready for ligation to the vector (as described below the pTVkIg-1 vector is digested with HindIII and the ends are made blunt by treatment with the Klenow fragment). The Ngf2 oligonucleotide (SEQ ID NO:76) alters the nucleotide sequence at the carboxy-terminal end of the protein to create an NgoMI restriction site near the 3' end of the NGF gene; this alteration changes the native (i.e., naturally occurring) sequence of AGGA at nucleotides 703 to 706 in SEQ ID NO:74 to CGGC. This change does not alter the amino acid sequence of the NGF protein in the final construction (see below) but adds a restriction site which aids in the cloning of the desired synthetic linker encoding a hydrophilic spacer and endoprotease site.

DETD The NGOKP1 and NGOKP2 oligonucleotides are annealed together at a concentration of 1 .mu.M (each) in 50 .mu.I TE (pH 8.0), 50 mM NaCl by heating to 85.degree. C. and slow cooling to room temperature over 2 hours. The resulting linker/adapter is ligated to the NgoMI digested **proNGF** PCR product to prepare the PCR product for insertion into the pTVkIgG-1 bacterial expression vector. The ligation of the synthetic linker/adapter to the NgoMI ends on the **proNGF** PCR product regenerates the original amino acid sequence at the carboxy-terminus of the NGF protein. The linker/adapter also truncates the natural dipeptide (Arg-Ala at position 109-110 in FIG. 31) that is not present on the mature product.

DETD The prepared insert (blunt-**proNGF**/linker/adapter-KpnI) is mixed with the prepared pTVkIgG-1 vector at a 3:1 (insert:vector) ratio in a 20 .mu.l volume comprising 1.times. T4 ligase buffer (NEB), 50 .mu.M ATP, T4 DNA ligase (200 units) is then added and the reaction is

[illegible] isolated using standard techniques and digested with NcoI and SmaI to identify clones with a single insert in the proper orientation. Positive [illegible]

in Example 1a. Colonies containing plasmids having the desired insert (by restriction analysis) and which produce a high titer of IgG are sequenced to confirm that the inserted DNA encode the desired **proNGF** fusion protein.

DETD FIG. 33 provides a schematic map of the pTV-TH-NGF vector. The location of the trc promoter, the pho signal sequences, the **proNGF** sequences, the junction region, the IgG fragment, the ampicillin-resistance gene and the lac repressor (lacI.sup.q) gene are indicated. The direction of transcription is indicated by the use of arrows inside the circle.

DETD FIG. 34 shows the nucleotide and amino acid sequences present at the junction region in pTV-TH-NGF. Sequences present at the carboxy-terminal end of the NGF protein, the CPB terminator, the hydrophilic spacer/Kex2 protease site, and the amino-terminal end of the IgG fragment (the affinity domain) are indicated. As shown in FIG. 34, a leucine amino acid separates the hydrophilic spacer and the **arginine** residue which is present at the carboxy-terminus of NGF. This hydrophilic spacer separates the authentic carboxy-terminus from the KpnI-IgG Fc fragment. The carboxy-terminal sides of the **arginine** residues within the hydrophilic linker are both substrates for Kex2 (Lys-Arg, Arg-Arg) while the leucine residue provides a barrier to CPB digestion in order to generate authentic NGF with a final CPA digest.

DETD The above-described procedure (exposure of the Kex2-digested NGF protein to CPB-Sepharose) efficiently removes only the carboxy-terminal **arginine** and lysine. In preparation for CPA digestion, the pH of the sample is adjusted to 8.5 with NaOH after adding 1/10 volume 1 M ammonia carbonate, pH 8.5. Ten units of immobilized CPA (Sigma) is added to the sample for every .mu.mol of substrate present. The reaction is incubated for 3 hours at room temperature (25.degree. C.) with end over end rotation to insure adequate mixing of substrate with the immobilized matrix. The immobilized CPA is removed by filtration. This reaction can be monitored by the analysis of 200 .mu.l fractions by the ninhydrin reaction for released free amino groups as described above (Doi, et al., supra). The reaction is complete when a molar equivalent of leucine residues are released to generate authentic NGF. Additional chromatography steps (i.e., ion exchange, gel filtration, RP-HPLC and/or FPLC) may be employed to gain even higher purity of the recombinant NGF.

DETD As shown in FIG. 32, the mature form of human BDNF ends with a carboxy-terminal **arginine** residue and the carboxy-terminal amino acids contain only a portion of the furin motif (e.g., Arg-Gly-Arg). Like other proteins in this family, BDNF contains hydrophilic amino acids at its carboxy-terminus therefore additional consideration in the design of the hydrophilic spacer is needed. Because of the presence of internal dibasic (Lys-Arg) sites within the mature BDNF molecules (see small boxes shown in FIG. 32), it is not a candidate for the in vitro removal of the pro region from the fusion protein as was described above for NGF. Instead, the preproBDNF protein is expressed as a fusion with the IgG fragment; the BDNF and IgG domains are joined via a hydrophilic spacer and sequences which provide a recognition site for the endoprotease renin. The expression vector encoding the BDNF fusion protein is expressed in mammalian cells which produce high levels of furin (e.g., kidney and liver cell lines). This endogenous furin is used to remove the pro region form the BDNF fusion protein in vivo; the secreted fusion protein comprises the mature form of BDNF joined to the IgG affinity domain. The affinity domain is removed from the BDNF protein by digestion with renin and authentic BDNF is then generated by treatment of the renin-digested BDNF with carboxypeptidases.

DETD As shown below, the degeneracy of the codons allowed the creation of an MluI restriction site at the 3' end of the gene without altering the

[illegible] in pTTH-BDNF, sequences present at the carboxy terminal end of the BDNF protein, the hydrophilic spacer, the renin recognition site (site of cleavage is indicated by the arrow [illegible]

the hydrophilic spacer contains a leucine and three lysine immediately following the **arginine** residue which is present at the carboxy-terminus of BDNF. This hydrophilic spacer separates the authentic carboxy-terminus from the renin recognition sequence and the KpnI-IgG Fc fragment. The lysines provide a hydrophilic spacer that is resistant to carboxypeptidase Y digestion at pH 5.75 [Klarskov, Anal. Biochem. 180:28 (1989)], while the leucine residue provides a barrier to CPB digestion in order to generate authentic NGF with a final CPA digest.

DETD The buffer is then changed back to the ammonia carbonate buffer as described above and the sample is concentrated to 1 mg/ml using a Centricon-3 cartridge (Amicon) for the CPA digestion. The sample is incubated with immobilized CPA (2 units/ml substrate) for 180 minutes as described above to remove the leucine and lysine residues that remain after the CPD-Y flow digestion. This reaction stops at the **arginine** residue at the carboxy-terminal position of authentic BDNF. Released amino acids are separated from authentic BDNF by gel filtration through a Sephadex G-25 column. Additional chromatography steps (i.e., ion exchange, gel filtration, RP-HPLC and/or FPLC may be employed to gain even higher purity of the recombinant BDNF.

DETD ---

SEQUENCE LISTING

- (1) GENERAL INFORMATION:
- (iii) NUMBER OF SEQUENCES: 90
- (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:

#acids (A) LENGTH: 8 amino
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

- (ii) MOLECULE TYPE: peptide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
- Asp Tyr Lys Asp Asp Asp Asp Lys

CLM What is claimed is:
3. The fusion protein of claim 1, wherein said susceptible amino acids of said hydrophilic spacer are selected from the group consisting of **arginine**, cysteine and lysine.

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DETD NT-4 nucleic acid is RNA or DNA which encodes a NT-4 polypeptide or which hybridizes to such DNA and remains stably bound to it under stringent conditions and is greater than about 10 bases in length; provided, however, that such hybridizing nucleic acid is novel and unobvious over any prior art nucleic acid including that which encodes or is complementary to nucleic acid encoding NGF, BDNF, or NT-3. Stringent conditions are those which (1) employ low ionic strength and high temperature for washing, for example, 0.15M NaCl/0.015M sodium citrate/0.1% NaDodSO.sub.4 at 50.degree. C., or (2) use during hybridization a **denaturing** agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42.degree. C.

DETD The third group of variants are those in which at least one amino acid residue in NT-4, and preferably only one, has been removed and a different residue inserted in its place. An example is the replacement of **arginine** and lysine by other amino acids to render the NT-4 resistent to protcolysis by scrinc protcascs, thcrcby crcating a variant of NT-4 that is more stable. The sites of greatest interest for substitutional mutagenesis include sites where the amino acids found in BDNF, NGF, NT-3, and NT-4 are substantially different in terms of side chain bulk, charge or hydrophobicity, but where there also is a high degree of homology at the selected site within various animal analogues of NGF, NT-3, and BDNF (e.g., among all the animal NGFs, all the animal NT-3s, and all the BDNFs). This analysis will highlight residues that may be involved in the differentiation of activity of the trophic factors, and therefore, variants at these sites may affect such activities. Examples of such sites in mature human NT-4, numbered from the N-terminal end, and exemplary substitutions include NT-4 (G.sub.78 .fwdarw.K, H, Q or R) (SEQ ID NOS. 13, 14, 15 and 16, respectively) and NT-4 (R.sub.85 .fwdarw.E, F, P, Y or W) (SEQ ID NOS. 17, 18, 19, 20 and 21, respectively). Other sites of interest are those in which the residues are identical among all animal species'BDNF, NGF, NT-3, and NT-4, this degree of conformation suggesting importance in achieving biological activity common to all four factors. These sites, especially those falling within a sequence of at least 3 other identically conserved sites, are substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 1 under the heading of preferred substitutions. If such substitutions result in a change in biological activity, then more substantial changes, denominated exemplary substitutions in Table 1, or as further described below in reference to amino acid classes, are introduced and the products screened.

DETD If the signal sequence is from another neurotrophic polypeptide, it may be the precursor sequence shown in FIG. 2 which extends from the initiating methionine (M) residue of NT-2, NT-3, or NGF up to the **arginine** (R) residue just before the first amino acid of the mature protein, or a consensus or combination sequence from any two or more of those precursors taking into account homologous regions of the precursors. The DNA for such precursor region is ligated in reading frame to DNA encoding the mature NT-4.

DETD Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione

the **arginine**

DETD Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, [illegible] **arginine**

Properties, W. H. Freeman & Co., San Francisco, pp. 79-86) acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. NT-4 also is covalently linked to nonproteinaceous polymers, e.g. polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the manner set forth in U.S. patent application Ser. No. 07/275,296, now abandoned, or U.S. Pat. Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

DETD Therapeutic formulations of NT-4 are prepared for storage by mixing NT-4 having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences, supra), in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone, amino acids such as glycine, glutamine, asparagine, **arginine** or lysine; monosaccharides, disaccharides and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or PEG.

DETD Aliquots of 200 .mu.l are taken from each 1 ml fraction collected, dialyzed against 1M acetic acid, lyophilized, and redissolved in 30 .mu.l Laemmli SDS-PAGE sample buffer (Laemmli, 1970, Nature 227:680). Human .beta.-NGF is obtained in a similar manner. Following SDS-PAGE, the silver-stained gel indicates a single, prominently stained polypeptide of approximately 15 kD. A 3-ml pool of S-300 column eluted fractions corresponding to this SDS-PAGE analyzed region is made, and 1 ml (0.5 nmole) is submitted to N-terminal amino acid sequence analysis by Edman degradation performed on a prototype automated amino acid sequencer (Kohr, EP Pat. Pub. No. 257,735). N-terminal sequence analysis gives a single sequence starting with a glycine residue predicted by the tetrabasic cleavage sequence ending in an **arginine**, and predicted by the processing of **preproNGF** to mature .beta.-NGF.

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